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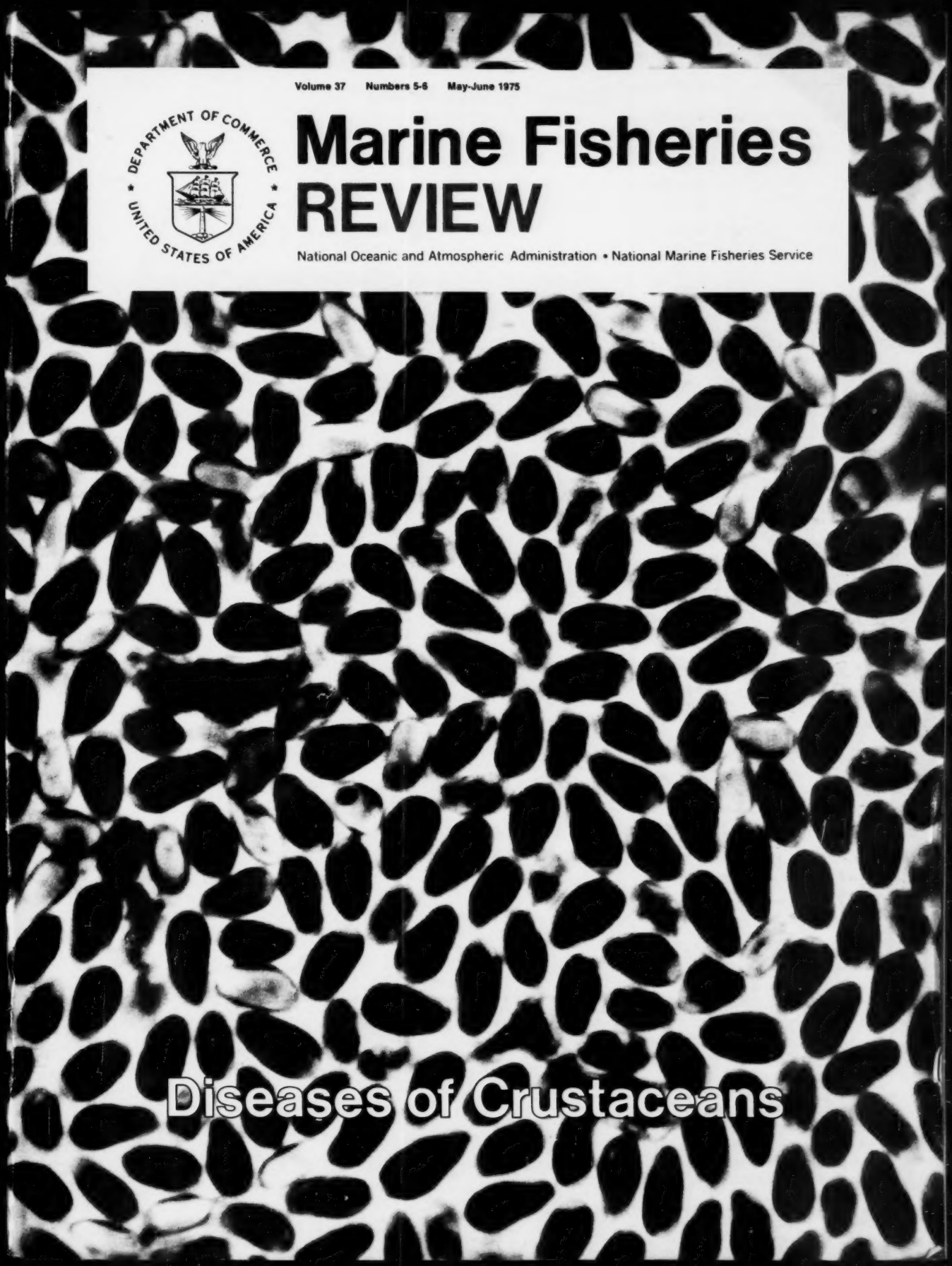

Diseases of Crustaceans

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Marine Fisheries Review

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Diseases of Crustaceans

Papers presented at the American Institute of Biological Sciences meeting, University of Massachusetts, Amherst, Mass. under the auspices of the Society of Invertebrate Pathology, 18-19 June 1973.

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Introductory Remarks on Diseases of Crustaceans

GILBERT B. PAULEY

Cultivation of several crustaceans (crabs, lobsters, shrimp, and crayfish) is carried out commercially and for sustaining natural resources. An understanding of crustacean pathogens, natural defenses of the animals against diseases, and methods of treating and preventing diseases will lead to more efficient and effective husbandry and ultimately better management of these resources. The objective of this Symposium was to collect a group of papers dealing with diseases of crustaceans. It seems to be a very propitious time to have this Symposium, because there is an ever increasing burden on the land and the sea to support the world's burgeoning population. Because of this increased burden, man must make more efficient use of the sea and its resources. In order to exploit economically the sea and the inland waterways, large numbers of fishes and shellfish must be cultivated in a relatively small volume of water. Under such unnatural crowding it is inevitable that inimical conditions, including diseases, will be encountered. At present economics dictate that gourmet items are the most profitable to raise. Therefore, the loss of even individual animals may become a serious matter. This is especially true as the animals grow larger and reach marketable size.

For infectious diseases to occur, a potential pathogen must exist, a suitable host must be present, and the proper environmental conditions must pervade that will cause either an increased virulence of the pathogen or an increased susceptibility of the host (Fig. 1, after Snieszko, 1973). Examination of the literature pertaining to cultured salmonids (salmon and trout) indicates that they are susceptible to a variety of serious diseases (Anderson and Conroy, 1968a; Cisar and Fryer, 1969; Christensen, 1972; Wood, 1968). The artificial stress conditions that include overcrowding, poor nutrition, reduced oxygen content, increased organic material, and pH and temperature changes are factors which increase the probability of disease among propagated fishes. Anderson and Conroy (1968b) indicate that several diseases may affect the aquaculture of English prawns (*Palaemon serratus*). Many of these probably are initiated by the numerous stresses placed upon the animals in the confinement of an aquaculture system.

Infectious diseases are found in both cultured crustaceans and those in the natural environment. At least four are serious diseases that occur in economically important crustaceans. Both bacteria and fungi are implicated in shell disease which is found in a variety of crustaceans, including crabs, lobsters,

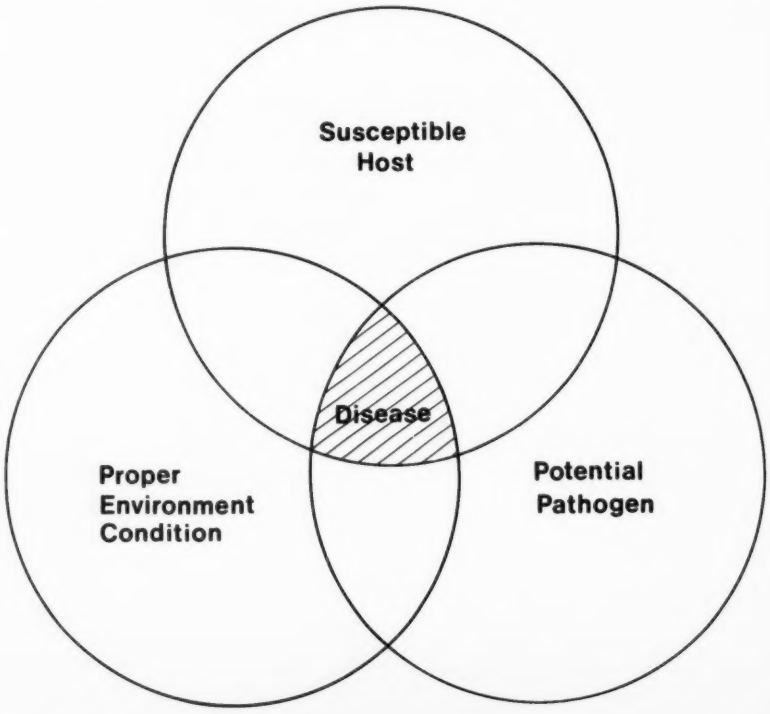

Figure 1.—Interaction of host, environment, and pathogen to produce a disease (Snieszko, 1973).

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and crayfish (Rosen, 1970). Gray crab disease, caused by a protozoan (*Paramoeba perniciosa*) is fortunately ephemeral and confined to the blue crab, *Callinectes sapidus* (Sawyer, 1969). The fungus, *Aphanomyces astaci*, has caused serious mortalities for many years among the European crayfish, *Astacus astacus* (Unestam and Weiss, 1970; Unestam and Nylund, 1972) and has been known to cause mortalities as high as 100 percent. At present, only one disease of the Crustacea has received adequate research attention and that is the fatal septicemia of lobsters caused by the bacterium, *Gaffkya homari* (Stewart and Rabin, 1970). The host-pathogen interactions are understood well in this disease.

In addition to infectious diseases, various noninfectious diseases may be important considerations in any crustacean aquaculture sytem. Sparks (1972) has listed several noncommunicable diseases of invertebrates that are caused by heat, cold, heavy metals, detergents, pesticides, and herbicides. Disease may also be caused by toxins produced by noncommercial animals and plants present in an aquaculture system (Sparks, 1972).

The host is capable of responding in a variety of ways to both infectious and noninfectious pathological agents. Recent interest in the immune mechanisms of crustaceans has shown that these animals respond to various disease agents in a manner different from vertebrates (Sindermann, 1971; Miller et al., 1972; Stewart and Zwicker, 1972; Pauley, 1973). They do not possess gamma globulin or a specific anamnesistic ability. However, they do possess a limited ability to discriminate between certain types of antigens. In many cases they are able to respond to disease agents much more rapidly than vertebrates. However, as in fishes and other invertebrates, the immune response of crustaceans is temperature dependent. An understanding of the immune mechanisms of crustaceans is important in the successful manipulation of cultured crustaceans. Increased knowledge of pathogen virulence and immunity in fishes has led to better control of their serious diseases. The use of vaccines (Fryer et al., 1972) and chemotherapy (Herman, 1970) are two important weapons in the prevention of fish diseases that have not been explored in crustaceans.

These observations illustrate the significance of disease in crustaceans under natural conditions and the hazards that may occur in any large-scale attempts to cultivate gourmet Crustacea. Although the study of pathology in these animals is limited to a rather small number of species, the following symposium papers will, hopefully, stimulate extensive studies of many crustacean diseases.

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MFR PAPER 1140

Cellular Response to Injury in Penaeid Shrimp

C.T. FONTAINE and D.V. LIGHTNER

ABSTRACT—A review of the cellular response to injury in marine shrimp of the genus Penaeus *is presented. Studies on the inflammatory response of penaeid shrimp have shown six cell forms that respond to injury. These include three forms of hemocytes, fibrocytes, phagocytic cells of the loose connective tissue, and fixed phagocytes lining blood sinuses.*

The cellular defense mechanism is dependent upon the activity of the hemocytes. One type of hemocyte apparently engages in encapsulating foreign material while the other two types phagocytize and eliminate foreign or necrotic material by migrating to the external surface. Later, additional phagocytosis is accomplished by "fixed" phagocytes in the loose connective tissue and by cells that line hemolymph sinuses of the heart, gill, and abdomen.

Collagen-like fibers are typically seen in association with wound healing and in the process of encapsulation in these animals. The dense network of collagen-like fibers and fibrocytes develops in close association with earlier hemocytic encapsulation or deposits. This fibrous tissue is stable, not resorbed, and remains as a permanent "scar."

INTRODUCTION

Within the past 20 years, the possibility of commercial culture of marine invertebrates has gained wide attention and interest, especially with the marine decapod Crustacea belonging to the genus *Penaeus*. On 20 August 1970 there were approximately 75 organizations in the United States involved in shrimp culture including federal, state, institutions, and companies or individuals. In addition, considerable information has been published relating to larval culture, tolerance studies, and pond culture of penaeid shrimp (Johnson and Fielding, 1956; Fujinaga, 1969; Allen, 1963; Zein-Eldin, 1963; Ewald, 1965; Zein-Eldin and Aldrich, 1965; Cook and Murphy, 1966; Zein-Eldin and Griffith, 1966; Cook, 1967; Wheeler, 1967; Zein-Eldin and Griffith, 1967; Aldrich et al., 1968; Cook and Murphy, 1968; Wheeler, 1968; Neal, 1970, 1970b; Mock and Murphy, 1970; and Lindner and Cook, 1971). The primary objective of the Aquaculture Investigation of the National Marine Fisheries Service, Galveston Laboratory, Galveston, Texas, has been to conduct investigations related to the culture of large numbers of penaeid shrimp. As in any intensive culture program, where conditions are also favorable for rapid proliferation of pathogenic organisms, one of the more important areas of consideration is the problem of disease.

The understanding of disease in any organism is dependent upon a basic knowledge of the organism's normal defense mechanisms. Cellular defense reactions have been reviewed in detail for insects by Salt (1970) and for invertebrates other than insects by Bang (1970) and Sparks (1972). A review of the literature, however, reveals very little information on histopathological investigations of marine Crustacea, particularly of the order Decapoda. The lack of recorded histopathological or normal histological data on marine decapod Crustacea is surprising, considering that several genera of this order

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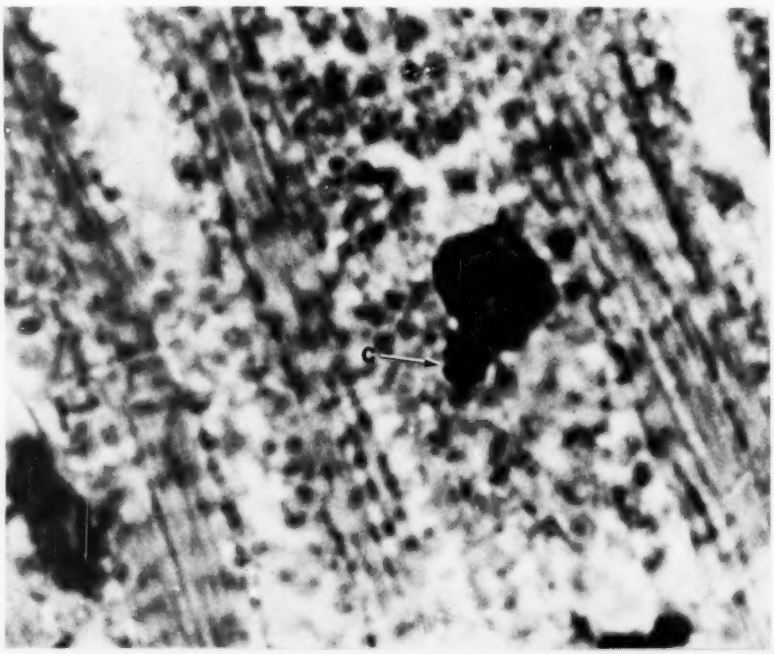

Figure 1.—A phagocytic form of hemocyte that has infiltrated the affected tissue and ingested foreign material (c = carmine). From Fontaine and Lightner, 1974. Hematoxylin, 2500×.

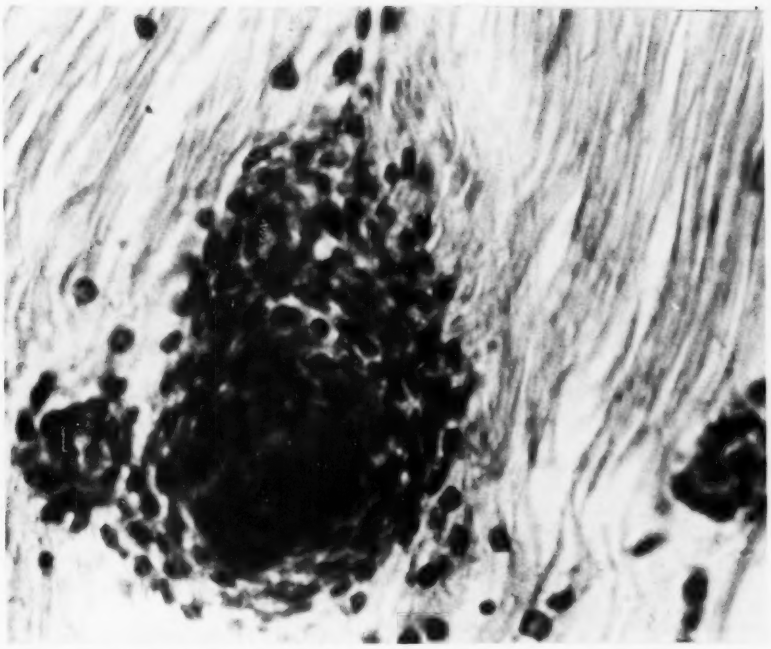

Figure 2.—A large hemocytic encapsulation. The innermost cells have become melanized forming a dark pigmented membrane. From Fontaine and Lightner, 1973. Hematoxylin and eosin, 800×.

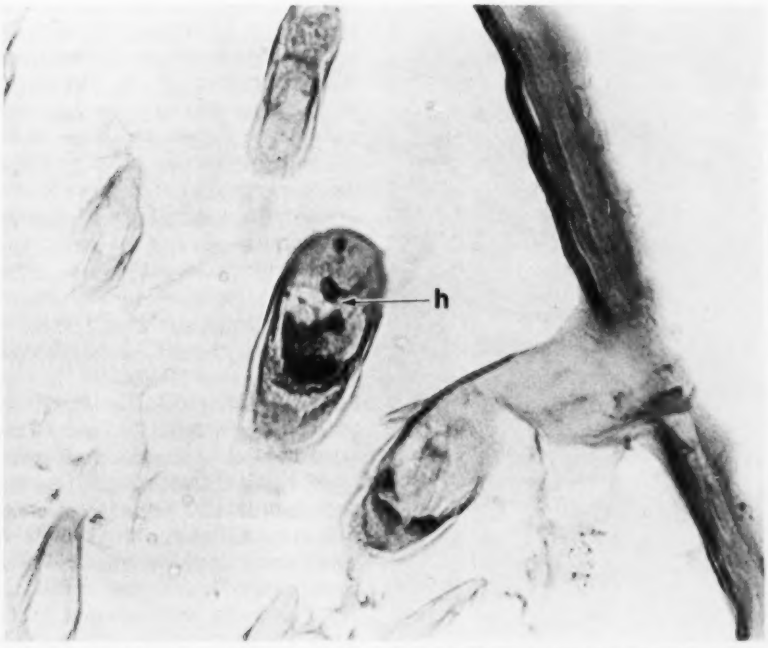

Figure 3.—The bases of setae with hemocytes included through which the phagocytes laden with foreign or necrotic material migrate to the external surface. (h = hemocytes). Hematoxylin and eosin, 650×.

are among the most economically important fishery products in North America. Prior to 1973, documented disease studies of penaeid shrimp were mainly from a parasitological viewpoint and contained little or no histological data (Aldrich, 1964; Iversen and Manning, 1959; Iversen and Van Meter, 1964; Kruse[1], 1959; Baxter, Rigdon, and Hanna, 1970; and Sprague, 1950, 1966). One exception was the histological study on "spontaneous necrosis" (Rigdon and Baxter, 1970).

Little is known about the humoral defenses or responses of penaeid shrimp. Foreign abiotic material injected into the abdominal musculature appears to adhere together to form extracellular clumps (Fontaine and Lightner, 1974). The factor in the hemolymph which causes this agglutination is not known. It is likely that this apparent humoral factor functions to localize the foreign material until cellular responses are initiated.

The subsequent cellular defense mechanisms in penaeid shrimp are dependent upon the activities of at least six cell forms. These include fibrocytes, "fixed" phagocytes in the loose connective tissue, and "fixed" phagocytic cells which line blood sinuses in the gill, heart, and abdominal muscle tissue.

HEMOCYTE FUNCTION IN PHAGOCYTOSIS

The hemocytes of penaeid shrimp typically migrate rapidly to invaded or injured tissue and, depending on particle size, engulf or encapsulate the necrotic or foreign material. Phagocytosis by hemocytes is accomplished by two cell forms that infiltrate the invaded tissue and ingest foreign or necrotic material. One form has eosinophilic cytoplasm and a small basophilic nucleus (Fig. 1). Occasionally, several of these cells that have phagocytized foreign or necrotic material will adhere together forming large multinucleated clumps that closely resemble the large multinucleated foreign body giant cells of other animal groups. A second form of hemocyte is a large macrophage-like cell that also infiltrates invaded tissue and exhibits phagocytosis. These cells possess a large, slightly basophilic nucleus with extensive hypochromatic cytoplasm.

HEMOCYTE FUNCTION IN ENCAPSULATION

Another function of circulating hemocytes is the encapsulation of foreign bodies that are too large for phagocytosis. It is interesting to note (Fontaine and Lightner, 1974) that when carmine particles were injected into the white shrimp, *Penaeus setiferus*, hemocytic encapsulations consisted

[1]Kruse, D. N. A study of the taxonomy, morphology, incidence and biology of the parasites of the commercial shrimp. *Penaeus aztecus* Ives, *P. duorarum* Burkenroad, and *P. setiferus* Linnaeus. M. S. thesis, Florida State University.

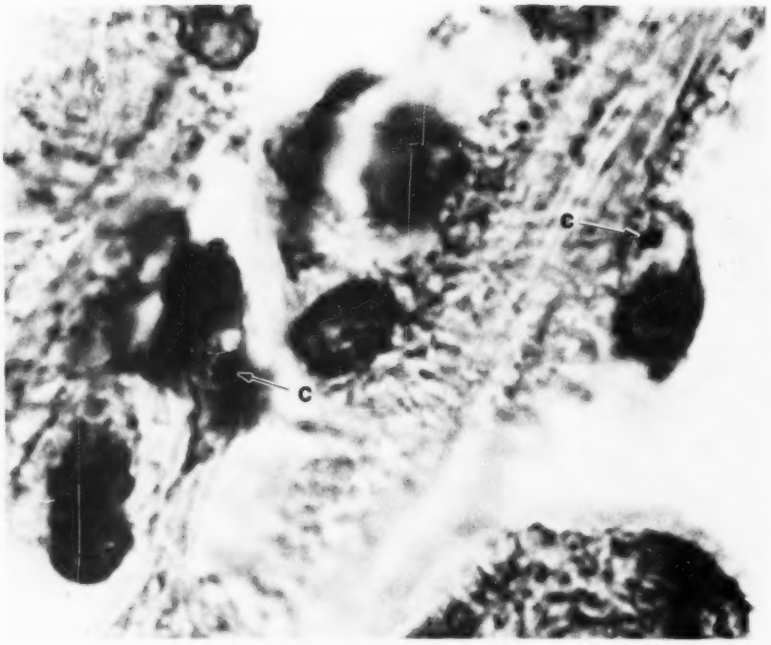

Figure 4.—Fixed phagocytes in blood sinuses of heart that have ingested foreign material (c = carmine). From Fontaine and Lightner, 1974. Hematoxylin, 2500×.

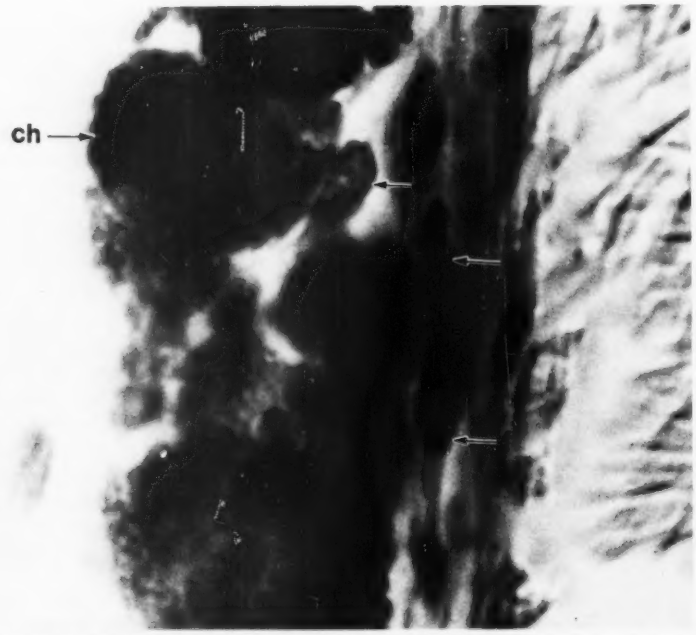

Figure 5.—Free and "fixed" phagocytes in an abdominal blood sinusoid (f = fixed phagocyte, c = carmine, h = free hemocyte, ch = carmine ingested by hemocyte). From Fontaine and Lightner, 1974. Hematoxylin, 2500×.

of necrotic hemocytes with carmine included. None of the cells actively engaged in the process of encapsulating these necrotic phagocytes were observed to contain carmine particles.

The mechanics of the process of encapsulation in penaeid shrimp are similar to that described for insects by Grimstone et al. (1967) and Salt (1970) with hemocytes many cell layers thick forming the encapsulations. The outer cells retain a more normal oval or rounded configuration, while the inner cells become flattened (Fig. 2) and, in later stages are lysed, forming a thick, brown, leathery capsule. These capsules are not resorbed and remain as marks of the foci of encapsulation even though no recognizable hemocytes remain.

The intensity of the cellular response in the process of encapsulation is variable. For example, the response to parasites depends upon the species of parasite and its location within the shrimp. The parasitic nematode, *Contraceacum* sp., elicits little or no cellular response from the shrimp no matter where it is located in the shrimp's body, while the pleuocercoid larvae of the cestode, *Prochristianella penaei*, is destroyed by encapsulating hemocytes if located within the hepatopancreas or muscle. The response to the larval cestode is much less intense if located in the hemocoelic space (Sparks and Fontaine, 1974).

HEMOCYTE ELIMINATION OF FOREIGN MATERIAL

One of the primary sites of elimination of foreign and necrotic material from the shrimp is the gills, with the actual elimination probably occurring with the molting of the gill cuticle. Within 1 hour after injection of carmine particles into shrimp, the lumens of the gill filaments become congested with the particles that are free in the hemolymph. Later, the particles are concentrated into large hemocytic clumps in the gill lamellae.

Another route of elimination of hemocytes laden with foreign or necrotic material appears to be through cuticular pores at the base of setae on the appendages (Fig. 3). The connective tissue and muscle at the base of the setae become congested with hemocytes. Many of these phagocytes have been seen located between epidermal cells of the cuticle. Necrotic hemocytes with carmine included have also been observed on the external surface in close proximity to or adhering to the setae. The great numbers of hemocytes observed in the maxillae, pereiopods, and pleopods where setae are present indicate these areas may be principal sites for eliminating hemocytes laden with necrotic or foreign material.

"FIXED" PHAGOCYTES

The hemolymph chambers or sinuses of the penaeid shrimp are lined with a

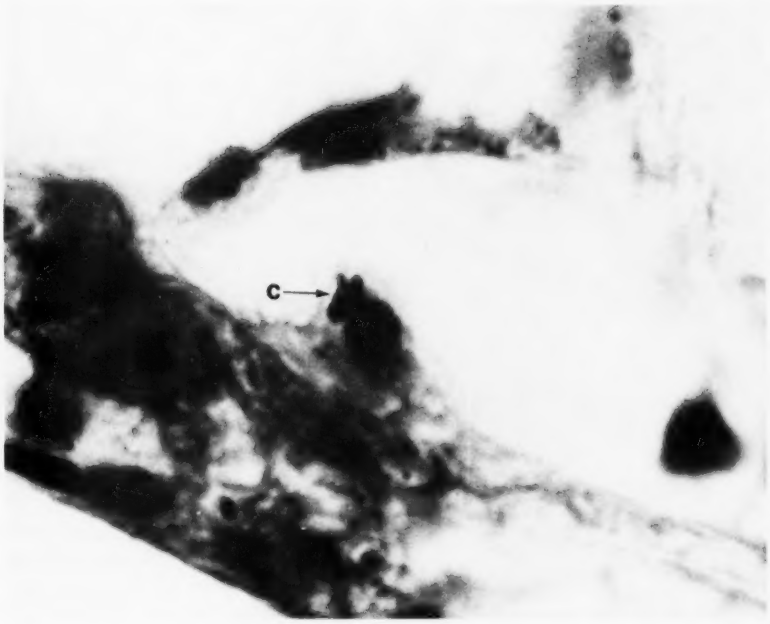

Figure 6.—A "fixed" phagocyte in a gill filament that has ingested foreign material (c = carmine). From Fontaine and Lightner, 1974. Hematoxylin, 2500×.

network of "fixed" phagocytic cells. "Fixed" phagocytes containing ingested foreign material have been observed in the heart (Fig. 4), abdomen (Fig. 5), and the lumen of the gill filaments (Fig. 6). This system of "fixed" phagocytic cells is very similar to that described in insects and may be analogous to the reticuloendothelium of vertebrates (Wigglesworth, 1970). Other fixed phagocytes are the large basophilic cells of the subcuticular loose connective tissue, particularly in the posterio-dorsal portion of the cephalothorax (Fig. 7). These "fixed" phagocytic cells are apparently long lived. Fontaine and Lightner (1974) observed carmine included in phagosomes in these fixed cells 672 hr after injection. This system of fixed phagocytic cells thus accounts for the slow clearance rate of injected stains from penaeid shrimp (Neal, 1969).

FIBROCYTES

In penaeid shrimp wounded with a Petersen disk tag pin (Fontaine and Lightner, 1973), hemocytes infiltrate the area and wall off the pin, forming a dense, brown, leathery membrane. Later, the epidermis involutes into the wound, using the brown pigmented layer as basal support. The involuting epidermis then forms a chitinous layer similar in appearance to the normal external cuticle, thereby effectively relegating the pin to an external rather than an internal position. In association with the hemocytic response, large numbers of fibrocytes infiltrate the area and form a dense network of collagen-like fibers. It was shown in a subsequent study (Fontaine and Dyjack, 1973) that this fibrous tissue is not resorbed, is well organized, stable, and remains as a permanent "scar" (Fig. 8).

Another example of fibrocyte infiltration occurs in the replacement of tissues damaged by the injection of turpentine (Fontaine et al., In press). At 50 days post-injection, large fibrous cysts had been formed at the site of injection by infiltrating hemocytes and fibrocytes (Fig. 9). Heart tissue damaged by the circulating turpentine had been infiltrated by hemocytes and fibrocytes by 240 h post-injection and collagen-like fibers and cellular encapsulations had appeared (Fig. 10). In another study where a strip of polyvinyl chloride was inserted into the abdominal musculature of the white shrimp, *P. setiferus*[2], fibrocytes infiltrated and formed a thick fibrous capsule around the primary hemocytic encapsulation (Fig. 11).

[2]Unpublished study, "An electron microscopic study of capsule formation in penaeid shrimp," on file at the Galveston Laboratory, Gulf Coastal Fisheries Center, NMFS, Galveston, TX 77550.

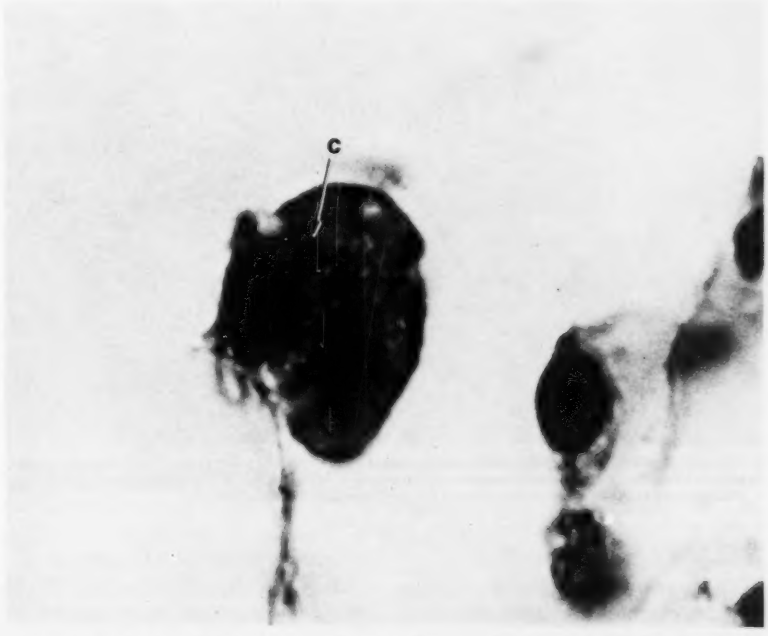

Figure 7.—A large basophilic cell in the subcuticular loose connective tissue of the cephalothorax with ingested foreign material (c = carmine). From Fontaine and Lightner, 1974. Hematoxylin, 2500×.

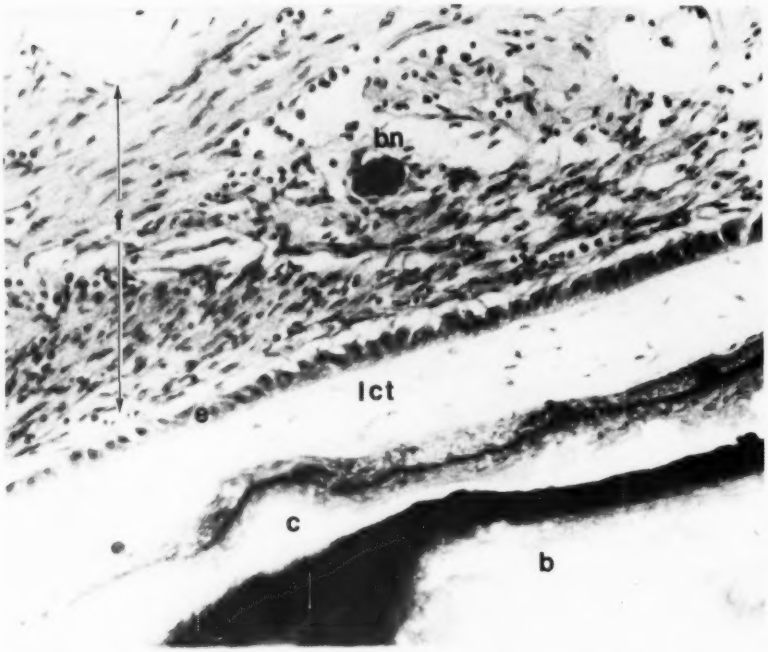

Figure 8.—The wound repair processes 30 days post-tagging with the Petersen disk tag (b = melanized membrane, c = cuticle, lct = loose connective tissue, e = epidermis, bn = brown nodule, and f = fibrous tissue). From Fontaine and Dyjak, 1973. Hematoxylin and eosin, 250×.

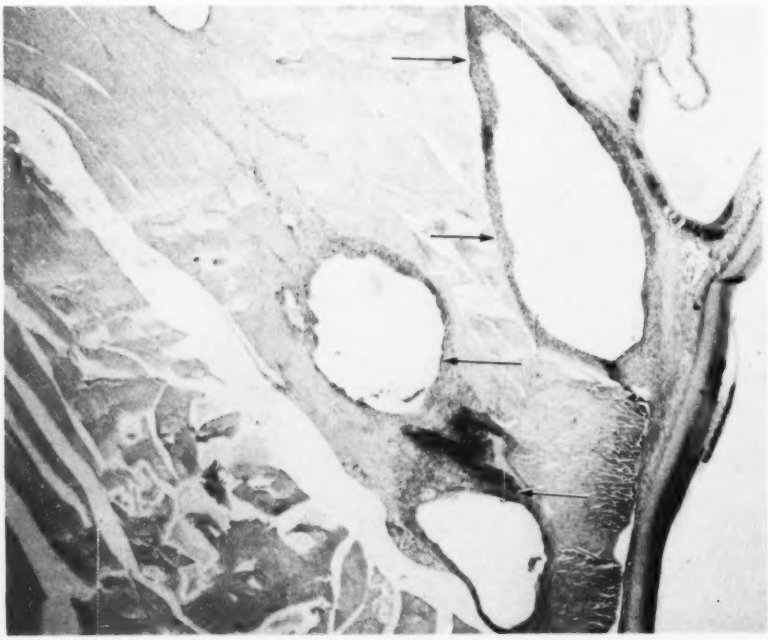

Figure 9.—The inflammatory response in *Penaeus setiferus* to turpentine injected into the abdominal musculature. The response consists of hemocytes and fibrocytes forming a thick matrix of fibrous tissue around the affected area (arrows). From Fontaine et al., in press. Hematoxylin and eosin, 40×.

MELANIN FORMATION

The brown material seen in association with hemocytes in several of the conditions in penaeid shrimp described in this paper has been reported to occur in other crustacea. The formation of a "black cap" from what appeared to be chitin on a severed appendage of the sand flea, *Gammarus* sp., was reviewed by Bang (1970). Sindermann (1971) described brown or "chitinoid" bodies or cysts in the gills as characterizing later stages of a number of crustacean diseases. A similar material has been shown to be melanin in insects (Salt, 1970). In their work on freshwater crayfishes, *Pacifastacus leniusculus* and *Astacus astacus*, Unestam and Nylund (1972) demonstrated conclusively that these decapod Crustacea do indeed form melanin in blood reactions in vitro. They also concluded that both the enzymes and the substrate for the process of melanization originate from the hemocytes. However, the formation of melanin following injury of penaeid shrimp remains to be proven. Dark pigmented material associated with hemocytes reacting to injury in shrimp is presumed to be melanin.

Shrimp which have undergone physiological stress from sudden or extreme temperature or salinity changes form numerous brown or black spots or nodules in the gill filaments. Larval shrimp invaded by the fungus *Lagenidium* sp. (Lightner and Fontaine, 1973) form brown spots in response to fungal hyphae in a manner similar to that described for the crayfish by Unestam and Nylund (1972). The pigmented spots have also been observed and recorded in association with bacterial erosion of the cuticle (Cook and Lofton, 1973; Lightner and Lewis, 1975); in wound repair (Fontaine and Lightner, 1973); after injection of carmine (Fontaine and Lightner, 1974), and turpentine (Fontaine et al., In press); in the capsular formation around an internal PVC tag[3]; and in an inflammatory response within a tumor (Sparks and Lightner, 1973). The black or brown spot syndrome occurs commonly (Fig. 12) and is a clinical sign of disease or injury that has been observed frequently in penaeid shrimp.

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Baxter, K. N., R. H. Rigdon, and C. Hanna.

[3]Unpublished data.

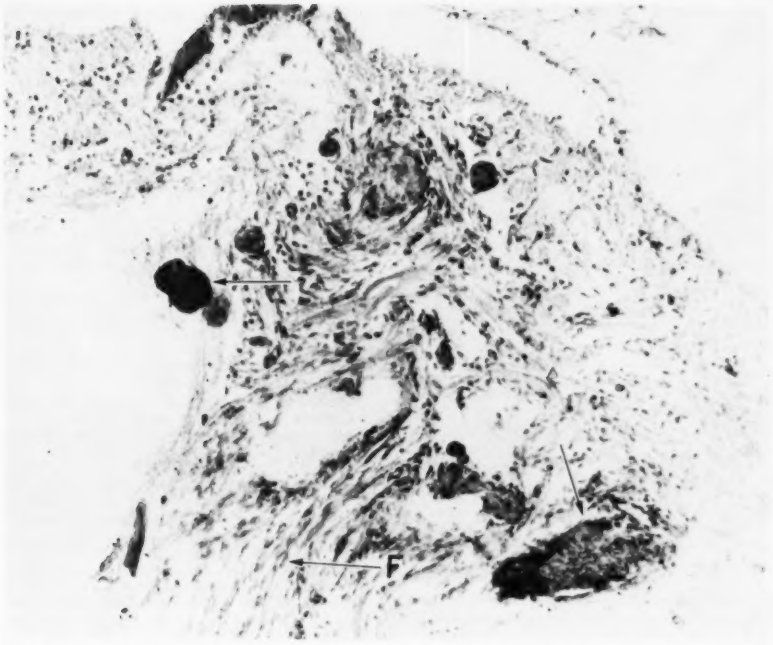

Figure 10.—The cellular response in the heart of *P. setiferus* damaged by turpentine. The response consists of hemocytic encapsulation (arrows) and many collagen-like fibers and fibrocytes (F). From Fontaine et al., In press. Hematoxylin and eosin. 60×.

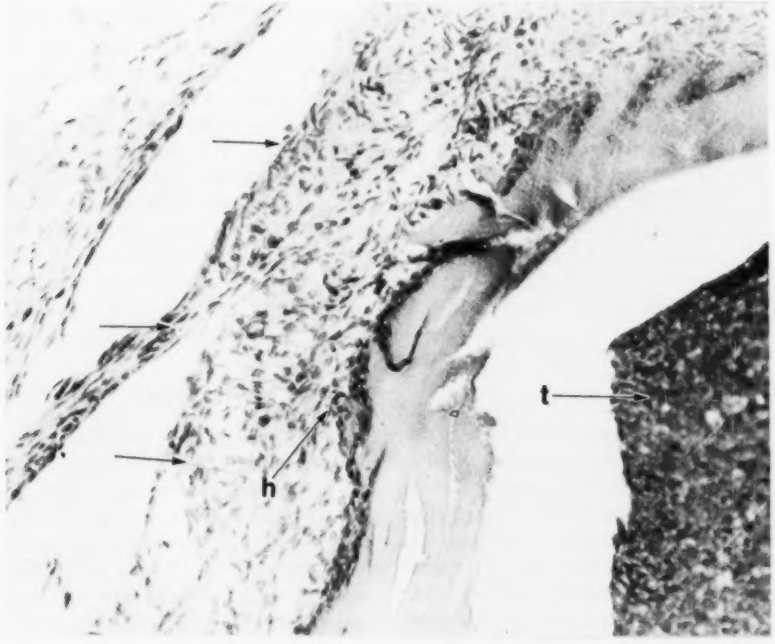

Figure 11.—The cellular response to an internal PVC tag inserted in the abdominal musculature of the white shrimp, *P. setiferus*. The fibrocytes have formed a fibrous capsule around the primary hemocytic encapsulation (arrow). t = plastic insert, h = hemocytes. Hematoxylin and eosin, 200×.

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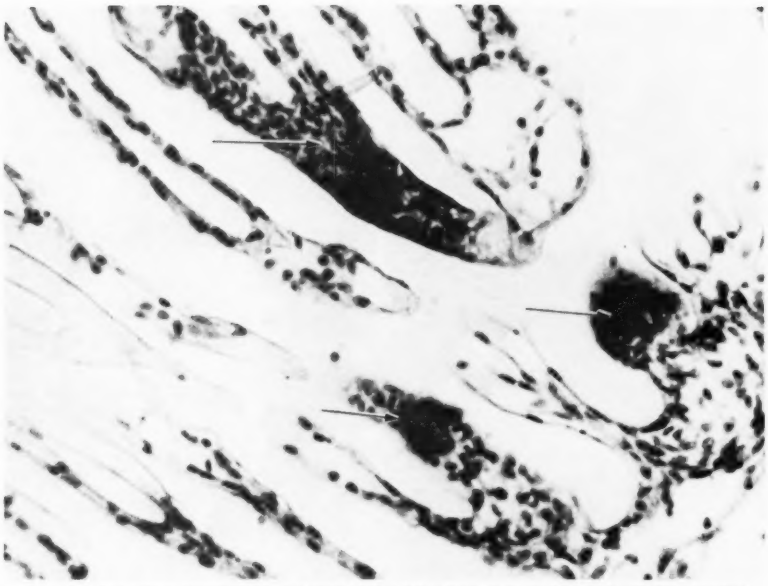

Figure 12.—The cellular response in gills of white shrimp 240 hr after injection of turpentine. These melanized spots or nodules (arrows) are a clinical sign of disease or injury in the penaeid shrimp. From Fontaine et al., In press. Hematoxylin and eosin, 250×.

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MFR PAPER 1141

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The Immunological Mechanisms of the Horseshoe Crab, *Limulus polyphemus*

JOHN I. STAGNER and JAMES R. REDMOND

ABSTRACT—The immunological system of Limulus polyphemus *is composed of a series of protein agglutinins which may be demonstrated to be distinct from one another by various immunochemical methods. The hemolymph agglutinates bacteria in vitro and inhibits bacterial growth. The levels of serum agglutinin vary after the injection of killed bacteria into* L. polyphemus. *The serum protein level falls after bacterial injection, but returns to normal within 12-24 h. The hemocyte hematocrit is directly proportional to the changes in hemolymph protein. Presumably the heteroagglutinin aids in removing bacteria from the hemolymph by preparing them for phagocytosis by hemocytes. Hemocyte lysates, which are endotoxin sensitive and remove endotoxin by gelatin, also agglutinate bacteria and inhibit bacterial growth in culture.*

A third and previously undescribed aspect of the immune system is a glycoprotein exudate produced by hypodermal glands and secreted through canals in the carapace. The exudate serves as a mechanical barrier to pathogens due to its viscosity and has agglutinating properties. The exudate is produced in response to injection of bacterial endotoxin or exposure to fouled seawater. Bacterial cultures are inhibited by the exudate. The exudate, like L. polyphemus *heteroagglutinin, immobilizes bacteria by binding the flagella or by rendering them inactive. RBC's and algae are also readily agglutinated by the exudate. During the secretory phase, large numbers of hemocytes move into the connective tissues under the carapace, around the hypodermal glands, and into the glandular ducts. The heteroagglutinin of the hemolymph, the hemocytes, and the exudate-producing glands provide an effective immunological defense against potential pathogens.*

INTRODUCTION

The anatomy and physiology of the horseshoe crab, *Limulus polyphemus*, has been examined in considerable detail. The attention this animal has received from the scientific community is justified on the basis that it is a very primitive arthropod. It is also the most primitive arthropod whose reactions to pathogens and injury have been studied. It has several immunological capabilities which have allowed it to survive from ancient times to the present. Its immune systems, which have been examined recently, are agglutinating proteins in the hemolymph and bacterial endotoxin reactive substances found in hemocytes. These two systems complement each other and function with a third, previously undescribed, system consisting of hypodermal glands which produce a viscous mucosubstance with agglutinating properties.

Natural humoral hemagglutinins are common among invertebrates and have been reported in most major phyla as discussed by Johnson (1968), Kahan and Reisfeld (1972), and Sparks (1972). The serum heteroagglutinin of *L. polyphemus* has been isolated and characterized by Cohen et al. (1965), Marchalonis (1964), and Marchalonis and Edelman (1968). In addition, Voightmann et al. (1971) and Sprenger and Uhlenbruch (1971) have shown that this heteroagglutinin is directed against N-acetylneuraminic acid receptors, certain carbohydrates, and basic proteins.

A great deal of research on the hemocytes of *L. polyphemus* has been performed (Bang, 1965; Levin and Bang, 1963, 1964, 1965, 1968; Young et al., 1972) which indicates the protein in hemocyte granules is coagulable upon exposure to bacterial endotoxin or to the lysis of hemocytes in wounds. The source of all coagulable protein in clotting reactions is apparently the hemocyte granules. Young et al. (1972) separated lysates prepared from hemocytes into two or three fractions. One fraction had an enzyme-like effect which increased the rate of gelation of a coagulable substrate fraction and could be activated by endotoxin. This reaction is very specific and has been used recently to detect gram-negative infections in humans (Rojas-Corona, 1969; Levin et al., 1970, 1972).

A previously undescribed facet of the immune mechanism of the horseshoe crab involves the carapace and hypodermal glands. The carapace, itself a mechanical barrier against invading pathogens, is penetrated by canals which serve as outlets for hypodermal glands. These canals have been reported by Karlson et al. (1968), but their function was unknown. This paper reports a description and characterization of the glands, the glycoprotein exudate, and their immunological role in *L. polyphemus*.

MATERIALS AND METHODS

Animal Collection and Maintenance

Animals were purchased from the Florida Marine Biological Specimen Company, Panama City, Fla., and maintained in Instant Ocean[1] aquarium systems and artificial sea salts at 15°C.

Bacterial Cultures

Cultures of *Pseudomonas atlantica* and *Vibrio marinopraesens* were purchased from the American Type

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Culture Collection, Rockville, Md., and were used for all agglutination and bactericidal studies, along with unidentified bacterial species cultured from *L. polyphemus* intestines. All bacteria were gram-negative and were grown on Difco marine agar throughout the study. Live bacteria were washed off slants with sterile seawater and diluted as required, or were killed by boiling for 30 min or exposure to 10 percent formalin in seawater overnight. The dead bacteria were centrifuged at 2,000 × g for 30 min and the pellet resuspended in sterile seawater and recentrifuged. This procedure was repeated three times. The killed bacteria were then stored in serum bottles at 4°C. The supernatant from boiling was retained and used as endotoxin.

Collection of Hemolymph

Hemolymph was collected by withdrawing blood into a precooled syringe fitted with a 20 gauge needle. The hemolymph sample was gently expelled from the syringe into a cold plastic centrifuge tube and centrifuged at 3,000 × g for 15 min at 4°C to remove hemocytes. Protein concentration in the hemolymph was measured with a hand-held refractometer calibrated with bovine serum albumin. The presence of the heteroagglutinin was determined by agglutination of RBC's (red blood cells) or bacteria.

Preparation of Hemocyte Lysates

Hemocyte lysates were prepared by centrifugation of the hemolymph as indicated above. The supernatant hemolymph was decanted and the pellet was washed by agitation in cold sterile seawater and centrifuged as discussed previously. The procedure was repeated three times and the resulting pellet was homogenized in a cold glass tissue grinder. Protein determinations were performed by the Folin-Lowry technique (Lowry et al., 1951) or spectrophotometrically at 280 nm using bovine serum albumin as a standard. Aliquots of lysate incubated with bacteria were observed for the removal of protein concomitant with agglutination of the bacteria.

Hemocyte Hematocrit Determination

Hemocyte numbers were determined by hematocrit. Siliconized capillary tubes were filled with precooled hemolymph, plugged with plasticine, and centrifuged 5 min in an International microcapillary tube centrifuge. In order to determine whether any change in hematocrit followed hemagglutinin change, hematocrits were determined at the same times as hemolymph protein concentrations.

Attempted Change of Hematocrit and Agglutinin Concentration

In order to induce and determine a change in the heteroagglutinin concentration in the hemolymph, animals were injected with 0.5 ml saline containing 5×10^6 heat- or formalin-killed bacteria per milliliter. Blood was drawn at 6 h intervals as previously described. The samples were divided to determine total protein, heteroagglutinin titer, and bactericidal activity. Secondary responses were produced by a bacterial challenge 36 h after the initial injection. To check for enhanced cellular numbers and activity, several horseshoe crabs were pre-immunized as described with 0.5 ml saline containing 2×10^5 bacteria per milliliter and hematocrit changes were followed at 6 h intervals.

Production of Exudate

Animals were injected with small amounts of endotoxin or were placed for several hours in fouled seawater prepared by allowing cod fish slices to spoil. The glycoprotein exudate was collected by pushing it to the edge of the prosoma with a rubber policeman and removing it with a Pasteur pipette.

Chemical and Physical Analysis of the Exudate

The protein moiety of the exudate was determined by absorbance at 280 nm or by the Folin-Lowry method (Lowry et al., 1951). The carbohydrate moiety was determined by the anthrone reaction (Bailey, 1958) or the phenol sulfuric acid reaction (Colowick and Kaplan, 1966). The Morgan-Elson reaction was performed on the exudate for the detection of glucosamine and N-acetylglucosamine (Morgan and Elson, 1934). Purification and fractionation was attempted with Sephadex chomatography using seawater as the eluant. Chemical and physical treatments of stability and function were carried out as outlined by Pauley et al. (1971) for: freezing and thawing, heat lability at 65° and 100°C; pH changes; pipetting; pre-adsorption with bacteria or RBC's; extraction with trichloroacetic acid, phenol, diethyl ether, and ethanol; and incubation with 2-mercaptoethanol. Samples were hydrolyzed in 10 N HCl for 24 h and run on a Beckman amino acid analyzer.

Exudate Agglutination Tests

Bacteria, algae, and human, dog, and rabbit RBC's were used for exudate agglutination. Agglutination tests were performed after each step in the physical and chemical tests outlined above. Protein and carbohydrate concentrations were examined after the addition of heat-killed bacteria to samples of the exudate. The mixture was incubated 4 h at 4°C, centrifuged at 2,000 × g for 30 min to remove bacteria, and then read for protein concentration at 280 nm and carbohydrate concentration by the phenol sulfuric acid reaction. Samples were dialyzed in either distilled water, seawater, or ethylenediaminetetraacetic acid (EDTA) decalcified seawater for 24 h and tested for agglutination with RBC's. Aliquots of tested samples were subsequently used for acrylamide gel electrophoresis. Phase microscopy was used to observe agglutination or phagocytosis of bacteria or RBC's. Hanging drop cultures of acellular hemolymph, cellular hemolymph, lysate, and exudate were prepared using depression slides and plastic or siliconized cover glasses. Bacteria or RBC's were added to a drop of the various samples and mixed by agitation of the slide preparation and examined immediately.

Electrophoresis

Acrylamide gel electrophoresis was performed by the method of either Davis (1964) or Hjerten et al. (1965a,b) using a 5 percent stacking gel and a 7 percent separating gel. Bromphenol blue was used as the tracking dye. Samples of 10-20 μl were layered on the surface of the stacking gel and run 1.5 h at 200 V and 2 A per tube. Samples of hemolymph, lysate, and exudate were run before and after treatment with RBC's or killed bacteria. The gels were stained for protein with amido Schwartz, and for glycoprotein with alcian blue, toluidine blue, and periodic acid Schiff (PAS) by the methods of

Maurer (1971). The molecular weight of the exudate was estimated by using sodium dodecyl sulphate (SDS) acrylamide gel electrophoresis by the method of Segrest and Jackson (1972).

Hypodermal Gland Anatomy and Histochemistry

Pieces of the dorsal surface of prosoma were fixed overnight in 10 percent neutral buffered formalin at 4°C, and then prepared for paraffin embedding. Other tissues were frozen in liquid nitrogen, freeze dried, formalin-vapor fixed, and paraffin embedded for histochemical staining. Some pieces also were embedded in methacrylate after the methods of Leduc and Bernhard (1967) and Rudell (1971) with dehydration in monomer at 4°C. Staining procedures were performed as outlined by Bancroft (1967) and Pearse (1968) for toluidine blue, mucicarmine, PAS, colloidal iron, alcian blue, azure A, low and high iron diamine alcian blue, periodic acidparadiamine, tetrazonium, Bial, Sakaguchi, 8-hydroxyquinoline, Millon reaction dimethylamino benzaldehyde performic acid-alcian blue, ninhydrin Schiff, and mercuric bromphenol blue. Specimens for scanning electron microscopy were frozen in liquid nitrogen, freeze dried, and coated with gold-palladium for observation of external surfaces. Samples were sonicated for cleaning, vacuum dried, and gold-palladium coated for the observation of internal surfaces.

Bactericidal Assays

The hemolymph was assayed for bactericidal qualities by the addition of 0.5 ml saline containing 5×10^3 bacteria per milliliter to various concentrations of serum, which were incubated 90 min at 20°C and then plated on marine agar. The same procedure was followed for samples of hemocyte lysate. Samples of exudate were centrifuged at 2,000 × g for 30 min to remove particulate matter and then placed in sterile culture dish halves and exposed to a germicidal ultraviolet lamp for 15 min. The samples were inoculated with an 18 h culture of *Vibrio marinopraesens* at a concentration of 5×10^3 bacteria per milliliter and incubated 1-4 h at 20°C before plating on marine agar. All plates were read 24 h after plating by comparing colony counts or areas with control plates.

Preparation of Antibody

Antibody was prepared against whole cell lysate, acellular hemolymph, and glycoprotein exudate by injecting rabbits subcutaneously with one of the materials mixed with Freunds complete adjuvant using the method of Clausen (1969). These immune sera were used to test for cross reactivity between the lysate, the hemolymph, and the exudate by using test tube precipitin and ring tests as outlined by Campbell et al. (1963) and Ouchterlony double diffusion tests.

HEMOLYMPH HETEROAGGLUTININ

The heteroagglutinin effectively agglutinates mammalian erythrocytes and certain bacteria as observed by phase microscopy. The material is heat labile and its action is calcium dependent. Heating at 65°C for 15 min destroys its activity as do pH extremes beyond pH 4 and pH 9. Compared to control plates, the heteroagglutinin inhibits bacteria growth as summarized in Table 1, and

Table 1.—Inhibition of bacterial growth by various concentrations of Hemolymph[1].

	Tube number									
	1	2	3	4	5	6	7	8	9	10
Colony number	0	0	0	2	4	2	5	10	30	103
	0	0	2	2	6	12	23	50	108	125
	0	3	4	16	12	14	18	18	26	89
	0	8	0	4	20	23	51	63	80	134
	0	0	0	0	11	18	35	67	93	100

[1]Tube numbers represent a dilution series of 1/10 with tube 10 as a bacterial control. A 0.05 ml/tube containing 5×10^3 bacteria was used throughout the experiment.

was most effective at high concentrations of hemolymph. Observations using phase microscopy suggest that bacterial flagella were bound or inactivated, rendering them nonmotile before death. Endotoxin ("0" antigen) prepared from *V. marinopraesens* also was agglutinated readily. These results indicate that the heteroagglutinin functions both as an agglutinin and as a bactericidin.

The level of heteroagglutinin was changed in the animal by the injection of killed bacteria. The protein concentration varied among animals, but always decreased about 6 h post-injection and rose above normal within 36 h. If the animals were reinjected at this time, the level fell and remained low for 24 h before returning to normal (Fig. 1). A decrease in the agglutination titer followed the decrease in hemolymph protein concentration and is presumably due to

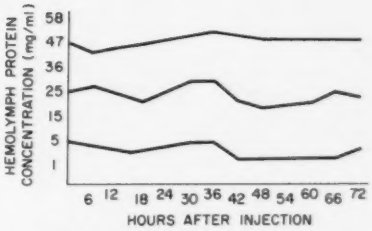


Figure 1.—Changes in hemolymph protein concentration after the injection of heat- or formalin-killed bacteria. The hemolymph protein concentration (mg/ml) differs between animals, as indicated in the three representative animals presented on graph.

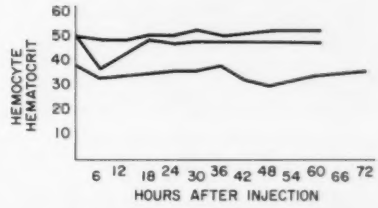


Figure 2.—Changes in circulation hemocyte hematocrits after the injection of heat- or formalin-killed bacteria. Individual variation of three animals is indicated on graph.

the immediate agglutination of injected bacteria which are cleared from the circulating hemolymph during this time. The origin of the heteroagglutinin is unknown.

HEMOCYTES AND COAGULATION

The injection of endotoxin into horseshoe crabs results in the coagulation of hemocytes, gelation of the hemolymph, and death apparently by asphyxiation due to intravascular clotting and paralysis. The animal becomes stiff and tetanic and the hemolymph is difficult to withdraw with an 18 gauge needle and syringe. This occurrence has also been noted by Bang (1956). The injection of killed bacteria causes a drop in circulating hemocytes for 6 h followed by a slow increase in cell numbers to about normal within 12 h (Fig. 2). The change in cell number parallels the change in hemolymph protein concentration. If an animal is bled repetitively 10 ml at a time and becomes stressed, the hematocrit increases markedly and the hemolymph becomes milky. The origin of the hemocytes is unknown, but from histological sections the storage areas appear to be in sinusoids located around muscles, the heart, the hepatopancreas, and gonadal

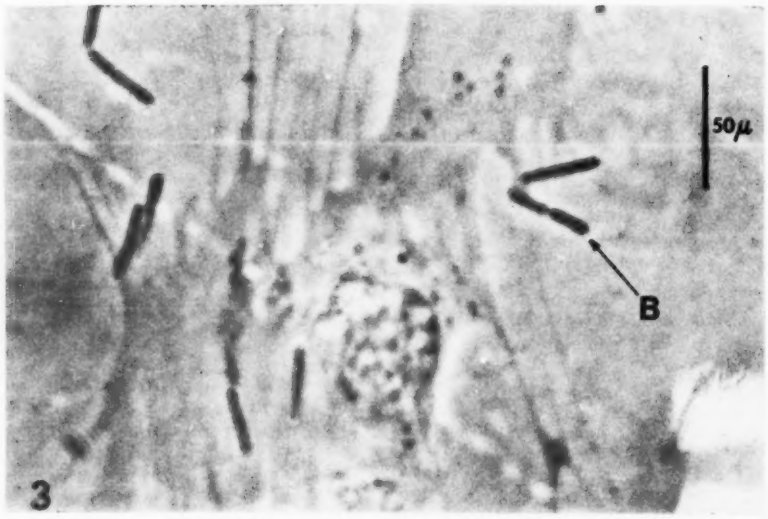


Figure 3.—The attachment of bacteria (B) to *Limulus polyphemus* hemocytes at the beginning of phagocytosis.

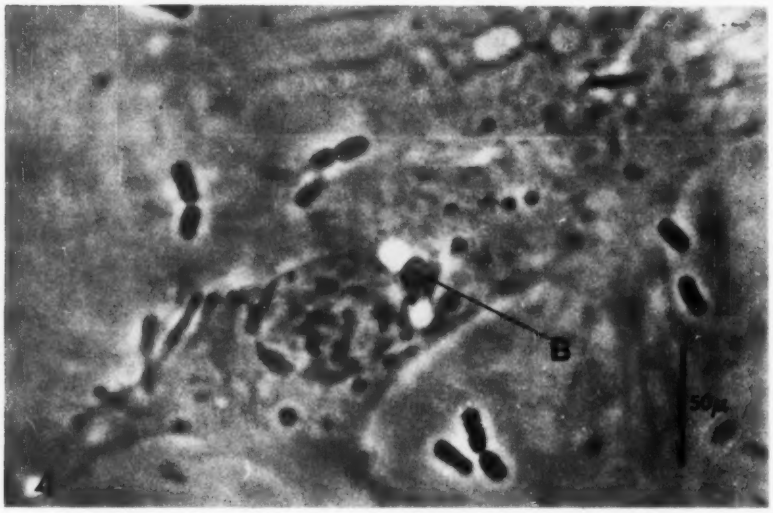


Figure 4.—The phagocytized bacteria (B) are contained within phagocytic vacuoles.

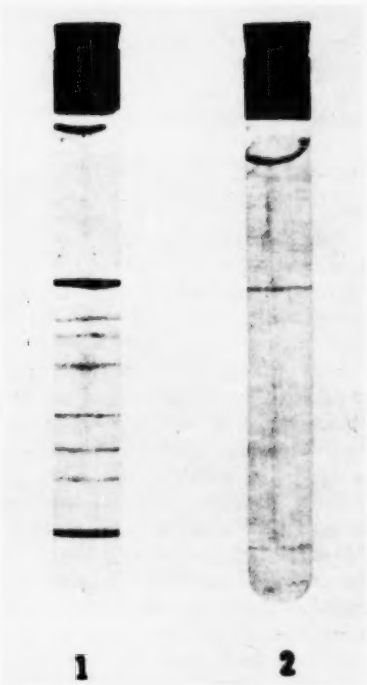


Figure 5.—Acrylamide gel electrophoresis of hemocyte lysate before (tube 1) and after (tube 2) the addition of bacterial endotoxin. Gels were stained with amido Schwartz.

tissues. Therefore, the replenishment of hemocytes is apparently due to their release from storage areas and is not due to an immediate increased production of cells. An injection of live bacteria may be successfully dealt with if the concentration of bacteria is below 2×10^5 per milliliter as reported by Smith (1964), since an LD_{50} represents approximately 10^6 bacteria per milliliter. No long-term enhancement of cell number or activity was found in immunization experiments with killed bacteria.

The hemocytes aggregate in vitro and form a fibrous clot with gel around it, which traps and immobilizes bacteria.

Table 2.—Inhibition of bacterial growth by various concentrations of lysate[1].

	Tube number									
	1	2	3	4	5	6	7	8	9	10
Colony number	0	0	0	0	0	0	3	55	78	200
	0	0	0	0	43	67	89	97	131	156
	0	0	0	0	0	18	39	53	80	140

[1]Refer to Table 1 for procedure.

This was observed also by Shirodkar et al. (1960) and Bang (1956). In hanging drop cultures of hemocytes, the cells degranulate and some agglutination occurs. Upon addition of bacteria, the agglutination of hemocytes is enhanced with bacteria attached at the periphery of the degranulated cells, which subsequently phagocytize the bacteria (Figs. 3, 4). Shirodkar et al. (1960) found that cells remained granular in culture, but degranulated with the addition of bacteria and became antibacterial. However, no phagocytosis was observed (Levin and Bang, 1968; Sparks, 1972).

The lysate agglutinated bacteria and inhibited bacteria growth (Table 2). Acrylamide gel electrophoresis demonstrated that normal lysate has two major protein bands with some minor staining bands between them. These lighter staining bands may be subunits of the other two darker, more pronounced bands, since column chromatography has shown the presence of only two groups of proteins. This has been corroborated by Young et al. (1972). Incubation with endotoxin or bacteria removes protein from the lysate and decreases the band densities as shown in Figure 5. This diminution of protein is due to its agglutination and precipitation by the endotoxin or bacteria. This reaction, like that of the heteroagglutinin, requires calcium ion and is effective at pH 6-8 with maximal activity at pH 7.5 (Levin and Bang, 1968; Young et al., 1972).

Figure 6.—Scanning electron micrograph of the inner surface of the dorsal prosomium of *Limulus polyphemus*. The indentations are muscle attachments (MA). Numerous canals are apparent.

HYPODERMAL GLAND MUCOPROTEIN

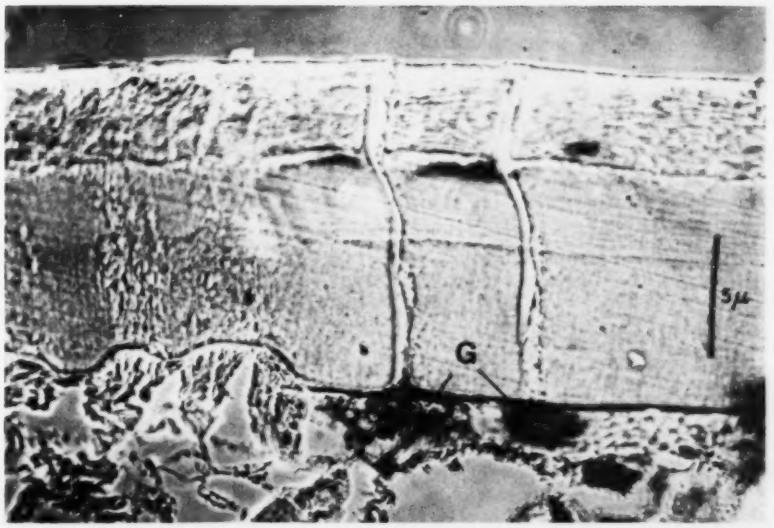
The histology of the carapace demonstrates the secretion pathways of the hypodermal glands through the carapace, with two types of canals penetrating the carapace (Figs. 6, 7). Canals housing gland ducts are $16 \times 22\,\mu$ and the larger canals are $55 \times 77\,\mu$. There are an average of 10-12 gland ducts for every large canal. These large canals are probably associated with sencilla on the dorsal surface of the carapace. Figure 8 shows the hypodermal glands and

Figure 7.—Scanning electron micrograph of the inner surface of the dorsal prosomium. The large canals (LC) are associated with sencillia and the small canals (SC) are associated with gland ducts.

Table 3.—Extraction procedures and inactivation tests to determine the stability and nature of exudate.

Experimental procedure	Complete inactivation of agglutinin	Agglutinin partially inactivated	Agglutinating activity unaffected
Physical treatments:			
Prolonged freezing (4-6 months)			+
Repeated freezing and thawing			+
Heat (65°C)	+		
Heat (100°C)	+		
Acidic pH extremes	+		
Alkaline pH extremes	+		
Dialysis:			
Distilled water	+		
Artificial seawater		+	
EDTA treated seawater	+		
Forceful pipetting			+
Preadsorption with bacteria or RBC	+		
Chemical treatments:			
Trichloracetic acid	+		
Phenol extraction	+		
Diethyl-ether extraction			+
2-mercaptoethanol incubation	+		
Ethanol	+		

Figure 8.—Section through the dorsal prosomium. Glands (G) with their associated ducts are seen to penetrate the carapace through canals.



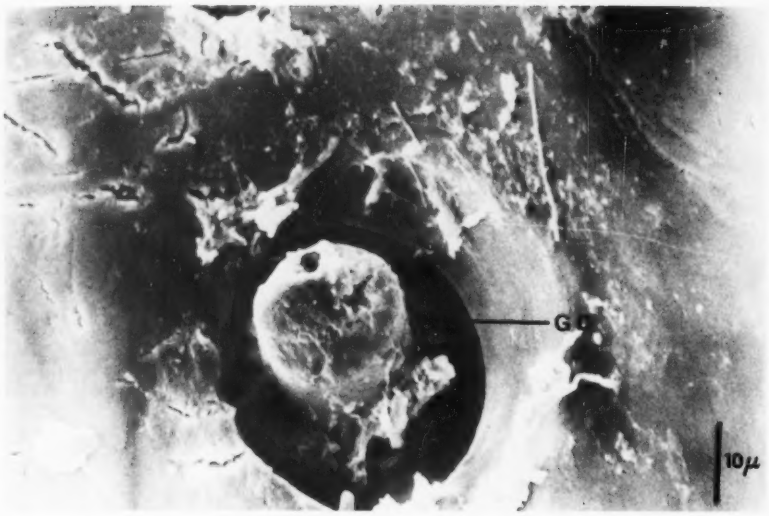


Figure 9.—Scanning electron micrograph of the outer surface of the dorsal surface of the prosomium. A gland duct (GD) is protruding from the outer opening of a canal.

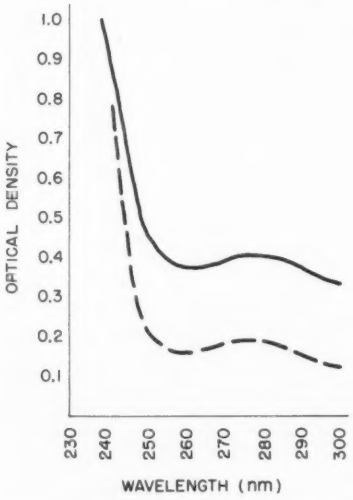


Figure 10.—Spectral absorption curve of exudate before and after the addition of killed bacteria. The solid line is untreated exudate and the broken line is bacteria treated exudate from the same sample demonstrating the adsorption of the protein agglutinin onto the bacteria.

Figure 11.—Acrylamide gel electrophoresis of exudate. Duplicate gels were stained for protein (tube 1) with amido Schwartz or carbohydrate (tube 2) with alcian blue.

Figure 12.—Acrylamide gel electrophoresis of exudate treated with killed bacteria. Tube 1 was stained with amido Schwartz and tube 2 with alcian blue, indicating bacteria adsorb and remove the agglutinin.

canals penetrating the carapace. The external ending of a duct may be seen in a canal opening (Fig. 9).

After stimulation, the production of viscous and elastic exudate persists for 12-24 h. Its presence may be evidenced by swirling a sample in a flask or stirring with a glass rod which produces spirals of the material in seawater. The exudate has both a protein moiety and a carbohydrate moiety and may contain 0.5-1.0 mg/ml of protein and 300-1,000 μg of carbohydrate per milliliter.

The function of the exudate is apparently twofold. It functions first as a mechanical barrier to pathogens due to its viscosity and secondly as an agglutinin. In vitro it readily agglutinates human, dog, and rabbit RBC's, algae, and both live and heat-killed bacteria. Its agglutinating ability was demonstrated by adding heat-killed bacteria or "O" antigen to samples of exudate and then reading the 280 nm absorbance before and after the incubation step. The bacteria adsorb protein from the hemolymph as shown in Figure 10, where 0.37 mg/ml of protein was adsorbed from an initial concentration of 0.82 mg/ml of protein. Hemolymph carbohydrate also shows a decrease of about 35 percent after treatment with bacteria or "O" antigen.

The material's viscosity may be lost irreversibly with repeated forceful pipetting, heating to 60°C for 15-30 min, and changes of pH beyond 7-7.6. Its activity is destroyed by heating, pH

changes, TCA (trichloroacetic acid), and alcohol treatments, but it retains its viscosity and function after freezing and thawing several times and may be frozen for several months. The material has not been separated effectively by Sephadex chromatography, since it binds to the Sephadex beads. A similar problem has been encountered by Ishiyama and Uhlenbruch (1972) with snail agglutinin, which had to be eluted from Sephadex with 0.5 M glucose. However, this technique was not successful when applied to horseshoe crab exudate. The exudate is dialyzable with distilled water, artificial seawater, and EDTA decalcified seawater. When dialyzed against artificial seawater, the materials retained and passed through the dialysis membrane are both active in agglutination. However, distilled water and EDTA treated artificial seawater effectively inactivate the exudate as tested with RBC's, indicating it is dependent upon calcium ions. A summary of chemical and physical treatments on the exudate is presented in Table 3.

Acrylamide gel electrophoresis of the exudate resulted in the separation of one or two protein bands and two carbohydrate bands (Fig. 11). The farthest migrating protein and carbohydrate bands approximate each other very closely. Incubation of exudate with RBC's or killed bacteria decreases or removes the protein bands and decreases the staining of the carbohydrate bands due to the adsorbance of these materials to the bacterial cell walls or RBC's (Fig. 12). Preliminary work using SDS acrylamide gel electrophoresis for molecular weight estimation suggests a molecular weight for the complex below, 6,000 daltons (one dalton equals the mass of one hydrogen atom). However, due to decreasing binding of SDS with glycoproteins an anomalous decrease in mobility may occur, so that molecular weights less than 10,000 may be only approximated by this method.

Histochemically the hypodermal glands are PAS positive and give metachromatic reactions for acid mucopolysaccharides as discussed by Pearse (1968) and Bancroft (1967). Pearse (1968) assumes this as evidence of sulfated mucosubstances which may contain hyaluronic acid or sialomucins. Tetrazonium reaction for sulfate groups are positive as are performic acid-alcian blue reactions for thiols and disulfides. Bial tests for sialic acid are also positive. Low and high iron diamine alcian blue staining and paradiamine staining also indicate the presence of sulfated mucosubstances with a content of uronic acid or sialomucin. Modified carbazole reactions for uronic acid and the Elson-Morgan reaction for Hexos-Protein stains are also positive. The reactions are summarized in Table 4 and indicate the presence of arginine, tyrosine, tryptophan, amino groups, and perhaps cysteine or thiol groups. Amino acid analysis shows the presence of proline, threonine, and aspartic acid. This indicates the exudate is a glycoprotein containing a small polypeptide and sulfated mucopolysaccharides made up of sialic acid, uronic acid, hexosamine, and perhaps hyaluronic acid.

After incubation in exudate, bacterial growth is inhibited and is most effective

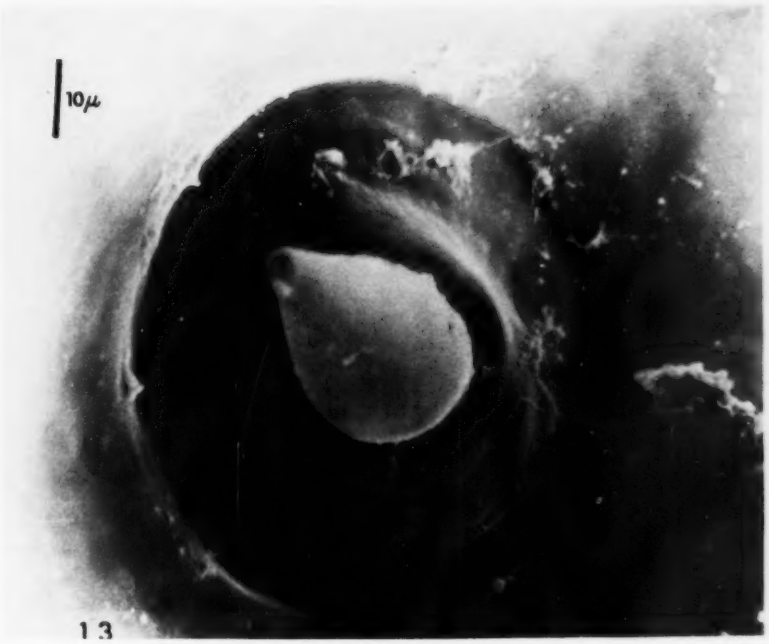


Figure 13.—Scanning electron micrograph of a sencillium on the dorsal surface of the prosomium.

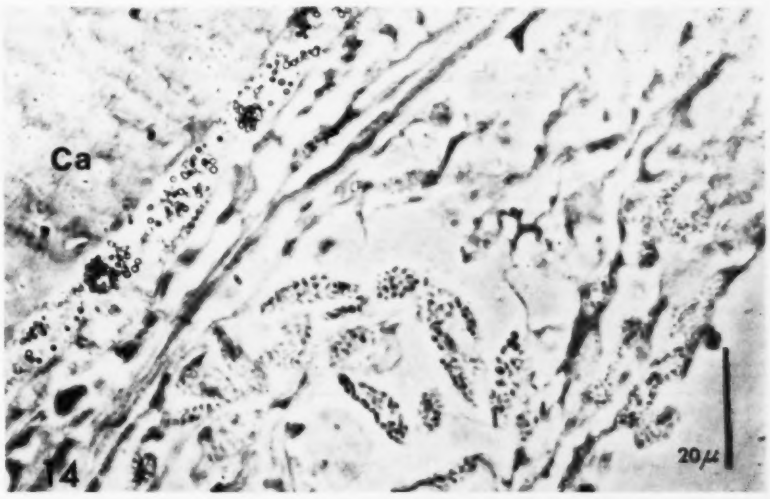


Figure 14.—A section through the dorsal prosomial carapace (Ca) demonstrating the presence of hemocytes in connective tissues.

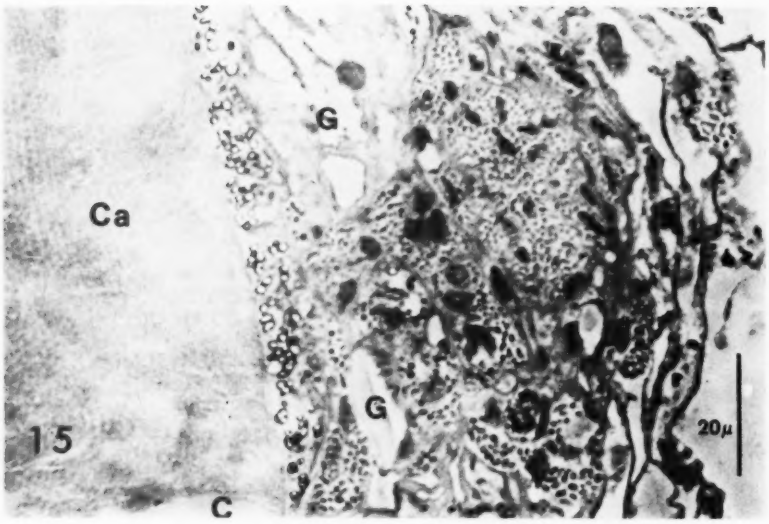


Figure 15.—A section through the dorsal prosomial carapace (Ca) demonstrating the presence of hemocytes in connective tissues and in association with glands (G) and canals (C).

Table 4.—Summary of histochemical staining reactions on hypodermal glands.

Method	Application	Result	Reference[1]
Toluidine Blue	Acid mucopolysaccharides	+	B
Mucicarmine	Acid mucopolysaccharides	+	B
PAS	Carbohydrates	+	B, P
Colloidal iron	Acid mucopolysaccharides	+	B
Alcian blue pH 1.0 and pH 2.5	Sulfated mucopolysaccharides Hyaluronic acid and sialomucins	+	B, P
Azure A pH 0.5 to pH 5.0	Sulfated mucopolysaccharides and sialomucins	+	B, P.
Low iron diamine Alcian blue	Sulfated mucopolysaccharides and sialomucins	+	B, P
High iron diamine Alcian blue	Uronic acid containing muco-polysaccharides and sialomucins	+	B, P
Periodic acid-paradiamine	Periodate reactive and neutral mucopolysaccharides	+	B, P
Tetrazonium	Sulfate esters and sulfonic acid	+	B, P
Bial	Sialic acid	+	B, P
Sakaguchi	Arginine	+	B, P
8-hydroxyquinoline	Arginine	+	P
Million reaction	Tyrosine	+	B, P
D.M.A.B.	Tryptophan	+	B
Performic acid-Alcian blue	Disulfides	+	B, P
Ninhydrin-Schiff	Amino groups	+	B, P
Mercuric-brom-phenol blue	Proteins	+	P

[1]Bancroft, 1967 (B) and Pearse, 1968 (P).

at short incubation times (Table 5). Observations with phase contrast microscopy show that the bacteria were agglutinated in clumps of 10-12 and were nonmotile after exposure to the exudate. These results suggest that the viscosity and the mechanical barrier action of the exudate are due to its carbohydrate moiety as discussed by Hunt (1970) and that its agglutinating property is due to the protein moiety directed against protein-polysaccharide complexes found on bacterial cell walls ("O" antigens) and membrane sites on RBC's. Since the results of the antigen-antibody test of cross-reactivity between heteroagglutinin, lysate, and glycoprotein exudate were negative, it is assumed that there is no serological relationship between these groups of agglutinating molecules although they are similar in action and antigenic sensitivity.

Table 5.—Inhibition of bacterial growth by hypodermal gland exudate[1].

Incubation time in hours	Experimental	Control
1	14.5	55.4
2	21.12	55.4
3	30.45	55.4
4	27.72	55.4

[1]Areas occupied by bacterial colonies were measured by planimetry and averaged for five samples of exudate.

The secretion of a protective glycoprotein by a marine arthropod has not been reported in the literature. This may be an adaptive mechanism of *Limulus polyphemus* and other primitive arthropods which has been lost through evolution or has been overlooked by investigators. Scanning electron microscopy and light microscopy reveal the presence of sencilla on the dorsal side of the carapace (Fig. 13). These structures are 19.2-48 μ in length and 22.8-29.5 μ in width. The presence or function of these structures has not been reported in the literature. However, chemoreceptors on the legs and gnathobases have been described by Hayes (1971). Perhaps these sensory structures are responsible for the detection of contaminants or endotoxin and trigger the secretion of exudate. Slightly before and during the active exudate secretion phase, a large number of hemocytes move into the connective tissues under the carapace, surround the hypodermal glands, and move into the canals of the glands and sencilla (Figs. 14, 15). This provides an effective connecting link among the several protective systems. In fouled or bacteria-contaminated water, exudate is initially produced, but if this and the carapace itself is penetrated, the heteroagglutinin of the hemolymph and the endotoxin sensitive phagocytic hemocytes are positioned to wall off or entrap invading pathogens.

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MFR PAPER 1142

Gaffkemia, the Fatal Infection of Lobsters (Genus *Homarus*) Caused by *Aerococcus viridans* (var.) *homari*: A Review

JAMES E. STEWART

Abstract—A review of recent literature is given concerning the fatal septicemia in lobsters caused by the bacterium, Aerococcus viridans *(var.)* homari *(formerly* Gaffkya homari*). Decreasing salinity may increase or decrease the time to death in lobsters, depending upon whether or not the animals have been infected before or after the salinity change occurred. As the disease progresses, glycogen, ATP, glucose, lactic acid, and nonprotein nitrogen all decrease. Impairment of the major metabolic processes is suggested as the probable cause of death. A degree of host resistance can be induced by administration of appropriate vaccines.*

INTRODUCTION

Gaffkemia is a fatal bacterial disease which periodically causes heavy mortalities among American and European lobsters (*Homarus americanus* and *Homarus vulgaris*, respectively). The disease and the causative agent, *Aerococcus viridans* (var.) *homari* (formerly *Gaffkya homari*)[1], were described originally by Snieszko and Taylor (1947) and Hitchner and Snieszko (1947). The septicemic nature of the disease and the taxon assigned to the pathogen prompted the name "gaffkemia" (Roskam, 1957) for the disease. Severe losses from gaffkemia are experienced periodically in commercial lobster units. Two recent reviews (Stewart and Rabin, 1970; Sindermann, 1971) have dealt in depth with this disease. The background material from these articles is summarized below. The causative agent, *A. viridans* (var.) *homari*, is a gram positive, catalase negative, beta-hemolytic, tetrad forming coccus which appears to possess no exoenzymes (Snieszko and Taylor, 1947; Hitchner and Snieszko, 1947; Hucker, 1957; Stewart et al., 1969b). This lack of exoenzymes and consequent lack of invasive powers results in the bacterium being transmitted only through breaks or ruptures in the integument of the host which permit entry to the hemolymph. The exposure to *A. viridans* (var.) *homari* must come simultaneously with or soon after integumental rupture for the pathogen to circumvent the lobster's rapid and effective wound healing mechanism. Transmission does not occur when the lobster ingests infected material since the acidity of the gastric fluid (pH 5.0) is lethal to *A. viridans* (var.) *homari*. Despite the pathogen's lack of invasive powers, the development of epizootics is aided by the aggressive behavior of lobsters, which provides wounds for entry, and by the fact that normal lobsters have no apparent resistance to the pathogen after entry. Small numbers of a virulent strain of *A. viridans* (var.) *homari*, consisting of 10 or less per kilogram of the lobster's weight, injected into the hemolymph are sufficient to ensure a fatal infection. Injection, however, of large numbers (6×10^8/kg) does not accelerate the infection sufficiently to produce a significant decrease in time to death. There are no external signs of the disease with the exception that as the infection proceeds the lobsters become lethargic and progressively weaker until they die.

As the disease develops, the lobster suffers a massive decrease of circulating hemocytes, resulting in impairment of the clotting mechanism by the removal of the clot initiating factor contained in the hemocytes. The fibrinogen levels and other hemolymph proteins are not affected significantly. As a result of the loss of hemolymph clotting power, the risk of a fatal hemorrhage is introduced in the event of wounding.

THE INFLUENCE OF ENVIRONMENTAL FACTORS

Variations in temperature and salinity have been examined and shown to have a marked effect on the disease. Since development of gaffkemia infections in lobsters is strictly temperature dependent, the disease remains largely dormant during the cold-water months. At 1°C the pathogen in vivo does not increase in numbers and the infection is subpatent until the temperature is increased. Then it flourishes and kills the host in a time span directly dependent upon the ambient temperature. The temperature influence on means for times to death (reported in days) were: 172 at 3°C, 84 at 5°C, 65 at 7°C, 28 at 10°C, 12 at 15°C, and 2 at 20°C. Additionally, the pathogen easily survives periods at 1°C, in vivo or in vitro, which are more than sufficient to carry the bacterium from one warm-water season to the next.

Salinity also has an influence on the course of the disease; the lobster, which is a relatively euryhaline animal, is a poor osmoregulator or conversely an "osmoconformer" (Dall, 1970).. Despite its osmoconformity, adaptations to salinity reductions occur slowly and require 75 h at 15°C for complete acclimation (Dall, 1970). It would be expected then that salinity change should in some way alter the pattern of the disease. The nature of the change, however, was quite unexpected (Stewart and Arie, 1973a). When lobsters were acclimated from the normal coastal salinity level of 31.8‰ to reduced salinity levels (26.1‰ and 21.5‰) prior to infection, the times to death upon infection were shortened in proportion to the salinity reduction (Fig. 1). Control animals exhibited no mortalities due to salinity changes. In contrast, when the salinity was reduced after infection an entirely different pattern developed. Reduction in salinity to 26.1‰ after

[1]See Stewart and Arie (1974) for review of name change.

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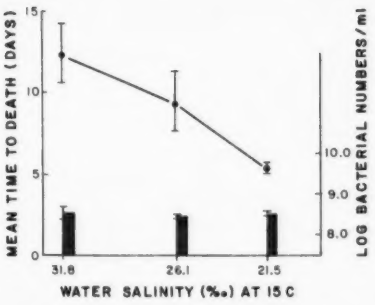

Figure 1.—Mean times to death (MTD) for lobsters infected with *A. viridans* (var.) *homari* and acclimated to water salinities of 31.8, 26.1 and 21.5‰ at 15°C prior to injection (——, MTD; bars indicate log bacterial numbers in lobsters). A total of 120 (60 infected and 60 control) animals were used in groups of 10 lobsters (4 groups at each salinity level). All groups were injected on the seventh day dated from the beginning of the salinity change. Each point represents the mean value plus standard error (SE) for a group of 20 infected lobsters. Uninfected control lobsters are not represented on this graph since none of these animals died (Stewart and Arie, 1973a).

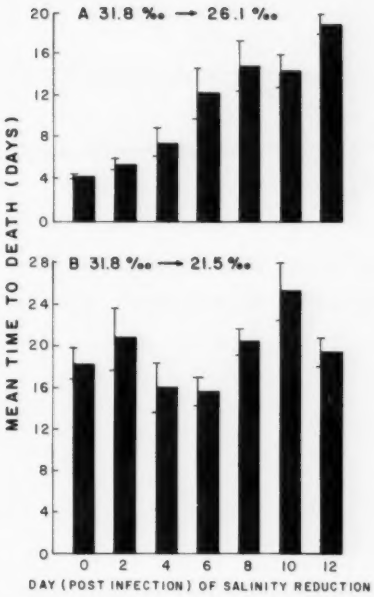

Figure 2.—Mean times to death for lobsters infected with *A. viridans* (var.) *homari* and subjected to water salinity reduction postinfection (over the course of 4 h) on the days designated. Columns represent mean values plus SE for groups of infected lobsters only, since none of the control animals died. (a) Reduction from normal 31.8 to 26.1‰. (b) Reduction of normal 31.8 to 21.5‰. Each vertical column represents a separate group of 10 lobsters plus SE (Stewart and Arie, 1973a).

infection initially decreased the times to death and then progressively increased the times to death (Fig. 2a), while reduction to salinity 21.5‰ after infection at any of the post-infection times increased the time to death (Fig. 2b) over that observed for infected lobsters kept throughout at salinity 31.8‰ (Fig. 1). An extensive series of experiments including sequential measurements of hemolymph nonprotein nitrogen, total carbohydrates, glucose, lactic acid, and serum osmolalities were performed during the course of the infections using both infected and healthy animals in an attempt to discover the basic reasons for the different responses (Stewart and Arie, 1973a). The growth of the pathogen in the infected animals subjected to the reduced salinities was examined, but it did not differ materially from that in infected animals kept at the normal salinity (Stewart and Arie, 1973a). Subjection of *A. viridans* (var.) *homari* to a much wider range of salinity values in vitro did not impair its growth (Stewart and Arie, 1973a). Although the data gained from measuring the various physiological parameters and the growth of *A. viridans* (var.) *homari* in relation to salinity change was interesting, particularly in terms of the responses of the noninfected control lobsters, none of this data nor a search of the literature suggested an explanation for the apparently contradictory results of the combined studies of salinity reductions and disease. Obviously the order in which the dual stresses of disease and salinity reduction are applied is a key factor in understanding the phenomenon. A point of departure for further studies might lie in a thorough examination of infected lobsters subjected to 26.1‰, where a decreased time to death occurs first, followed by a change in response resulting in increased times to death about the sixth postinfection day (Fig. 2).

PATHOGEN GROWTH AND BIOCHEMICAL CHANGES IN THE HOST

It has been concluded that death of lobsters infected with *A. viridans* (var.) *homari* is the result of an unsuccessful competition on the part of the lobsters for their own readily available energy reserves (Stewart et al., 1969a). The evidence for this is found in the pattern of development of the pathogen in vivo in the skeletal muscle, cardiac tissue, hepatopancreas and hemolymph (Fig. 3). Concomitant with the development of the pathogen is the decline of glycogen in the hepatopancreas (Fig. 4), the heart (Fig. 5), and the tail muscle (Fig. 6), and the loss of adenosine triphosphate (ATP) from these tissues (Figs. 7, 8, and 9). The ATP loss was most significant in the hepatopancreas. Parallel to these changes is the decline of the nonprotein nitrogen coincident with the disappearance of glucose and the decline of the lactic acid concentrations of the hemolymph (Fig. 10).

DISCUSSION

The development of the pathogen in vivo (Stewart and Arie, 1973b), the ability of the pathogen to utilize glucose, but not complex polysaccharides (Aaronson, 1956; Stewart and Cornick, 1972), its reliance on the nonprotein nitrogen (Stewart, Arie et al., 1969; Stewart, Foley, and Ackman, 1969) coupled with its inability to utilize proteins (Stewart, Arie et al, 1969; Stewart, Foley, and Ackman, 1969) indicate the following course of events. After entry into the hemolymph the pathogen is concentrated in the hepatopancreas and the heart, possibly as the result of phagocytic action. The subsequent development of bacteria in these two tissues is immediate and rapid, taking place at the expense of free glucose and nonprotein nitrogen. The latter is probably present in greater concentrations in the hepatopancreas than in the heart, which might account for the difference between the final bacterial numbers in these two tissues. As the bacterial numbers increase, especially in the hepatopancreas, the capacity of the phagocytes to transport the pathogen to and retain it in the hepatopancreas is lost through the increasing destruction of hemocytes, thereby permitting the pathogen to flourish in the hemolymph. This aspect of the interaction between the pathogen and the phagocytic mechanism is substantiated by the coincident exponential development of the pathogen in the hemolymph and the sharp decline in hemocyte numbers (Stewart, Arie et al., 1969). Glucose is the only fraction of the total carbohydrates in the hemolymph used by *A. viridans* (var.) *homari* (Stewart and Cornick, 1972; Stewart and Arie, 1973a) which the lobster can sustain at the expense of its glycogen. After exhaustion of the host's glycogen and subsequently glucose, the pathogen can utilize the limited supplies of lactic acid in vitro and apparently also in vivo (Stewart and Cornick, 1972; Stewart and Arie,

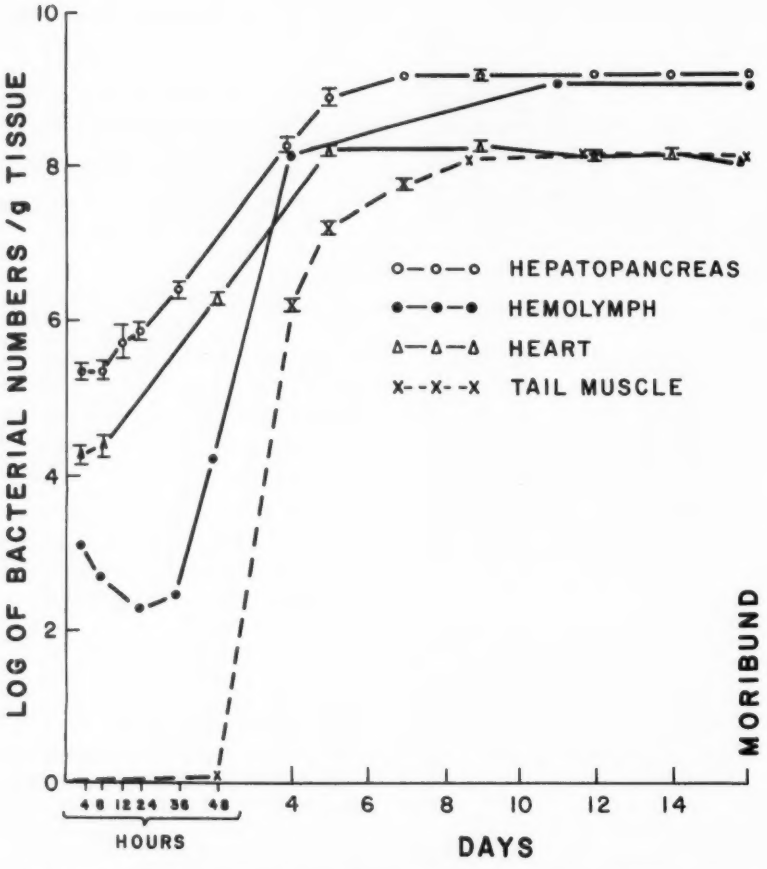

Figure 3.—Growth curves illustrating the development of *A. viridans* (var.) *homari* in the tissues of infected lobsters (Stewart and Arie, 1973b; Stewart, Arie et al., 1969).

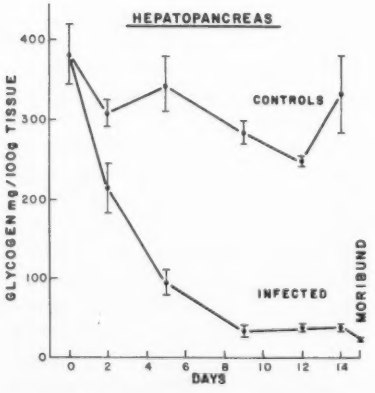

Figure 4.—Glycogen levels in the hepatopancreatic tissue of infected and control lobsters. Each point represents the mean value for five animals plus SE. The infected animals are those for which bacterial numbers are given in Figure 3 (Stewart and Arie, 1973b).

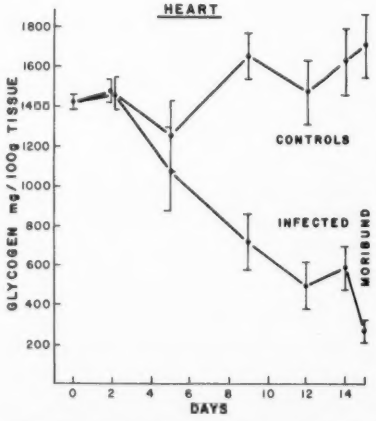

Figure 5.—Glycogen levels in the heart tissue of infected and control lobsters. The infected animals are those represented in Figure 3 (Stewart and Arie, 1973b). Each point plus SE represents a group of five lobsters.

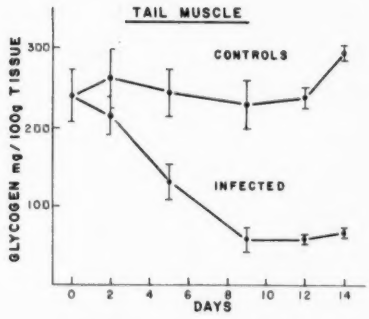

Figure 6.—Glycogen values in the tail muscle of the lobsters represented in Figure 3 (Stewart and Arie, 1973b).

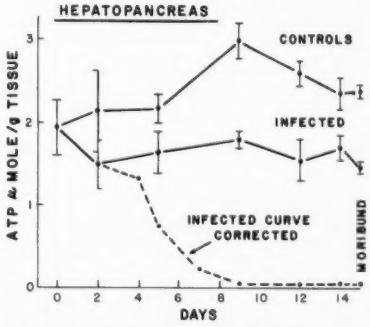

Figure 7.—ATP levels in the hepatopancreatic tissue of the lobsters represented in Figure 3 (Stewart and Arie, 1973b). The solid lines illustrate the actual measurements. The dashed line represents the corrected values obtained by subtraction of the bacterial ATP.

1973a). The drain upon the carbohydrate reserves in turn prevents the production of lobster hepatopancreatic ATP and suggests massive impairment of the vital hepatopancreatic functions of biosynthesis, detoxification, and repair (Stewart and Arie, 1973b). Lipid reserves remain unchanged throughout the course of the infection (Stewart, Arie et al., 1969), while hemolymph proteins, a ready reserve for the lobster (Stewart et al., 1967), are drawn upon only to a slight degree (Stewart, Arie et al., 1969). The possibility of replenishing the reserve materials from external sources is lost to the lobster by its refusal to feed after the onset of the infection. At the standard temperature of these studies, 15°C, lobsters will feed on the second day after being infected but refuse food thereafter (Stewart et al., 1972). The presumed reason for the refusal of food is the development of massive numbers of bacteria in the hepatopancreas, an organ which in crustaceans encompasses the function of liver and pancreas in vertebrates and also plays a prominent part in primary food absorption, a role fulfilled in vertebrates by the small intestine (Vonk, 1960). A soluble bacterial toxin would appear to be ruled out as a direct cause of death, since filter sterilized serum removed from heavily infected lobsters and then injected into healthy animals in amounts equal to more than 13% of the lobsters' body weights had no adverse

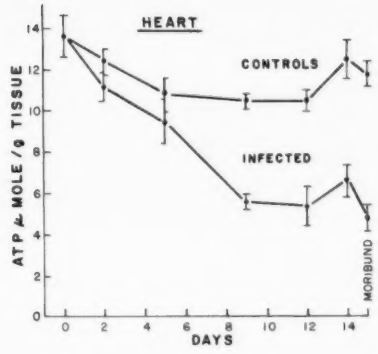

Figure 8.—ATP levels in the heart tissue of lobsters represented in Figure 3 (Stewart and Arie, 1973b). Bacterial ATP levels did not form a significant proportion of the total.

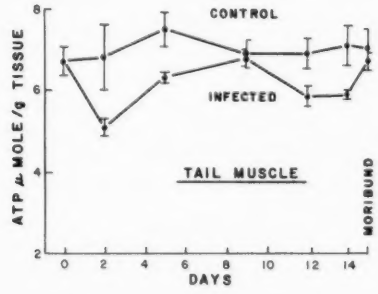

Figure 9.—ATP levels in the tail muscle tissue of lobsters represented in Figure 3 (Stewart and Arie, 1973b). Bacterial ATP levels did not form a significant proportion of the total.

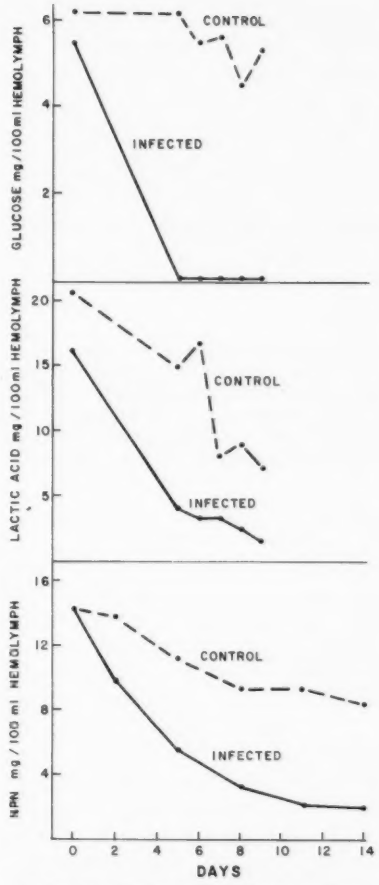

Figure 10.—Glucose, lactic acid and nonprotein nitrogen (NPN) changes in the hemolymph of infected and control lobsters (Stewart, Arie et al., 1969; Stewart and Arie, 1973a).

effect (Stewart and Arie, 1973b). Thus the extensive impairment of the central metabolic processes of the animal, which are carried out by the hepatopancreas, is suggested as the major cause of death in lobsters infected with *A. viridans* (var.) *homari*.

Resistance by lobsters to *A. viridans* (var.) *homari* infections has been observed only rarely (Stewart et al., 1966; Rabin and Hughes, 1968). It is possible, especially in the observations of Stewart et al. (1966), that the apparent resistance was actually due to a lack of virulence or a change in virulence of the pathogen. Under various influences, changes toward both increasing and decreasing virulence in *A. viridans* (var.) *homari* strains have been observed in this laboratory (unpublished results). Virulent strains of *A. viridans* (var.) *homari* are not affected by hemolymph agglutinins (Cornick and Stewart, 1968) or bactericidins (Stewart and Zwicker, 1972) and are able to overcome the effect of phagocytosis (Cornick and Stewart, 1968). A limited degree of resistance, however, has been observed upon injection of vaccines prepared from formalin-killed cells of avirulent strains of *A. viridans* (var.) *homari* (Stewart and Zwicker, 1974).

This resistance has taken the form of an increased time to death upon challenge with a standard suspension of a virulent strain of *A. viridans* (var.) *homari*, which is approximately double that recorded for animals challenged without being treated with the vaccine. The induced resistance has been shown to be quite separate from induced bactericidal activity (Stewart and Zwicker, 1974), a finding in keeping with results reported by McKay and Jenkin (1969) for induced resistance in the freshwater crayfish, *Parachaeraps bicarinatus*. The bactericidin in the serum of induced and noninduced lobsters, along with agglutinin activity and phagocytic action, however, does play a role in protecting lobsters against infection from microorganisms which otherwise could be as pathogenic as *A. viridans* (var.) *homari* (Cornick and Stewart, 1968; Stewart and Zwicker, 1972). The most recent studies on induction of resistance to *A. viridans* (var.) *homari* infections have resulted in the survival of challenged animals rather than mere increases in the time to death (Stewart and Zwicker, 1974b). These studies are being extended.

ACKNOWLEDGMENT

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MFR PAPER 1143

A Septicemic Bacterial Disease Syndrome of Penaeid Shrimp

DONALD V. LIGHTNER and DONALD H. LEWIS

ABSTRACT—A disease syndrome of penaeid shrimp characterized by the presence of a septicemic phase is described. Mortality due to the disease in infected shrimp populations typically ranged from 10 to 50 percent, but occasionally reached 100 percent. Vibrio alginolyticus, V. anguillarum, *and an* Aeromonas *sp. were isolated from moribund hatchery-reared white shrimp (*Penaeus setiferus*) and brown shrimp (*P. aztecus*) and from wild white, brown, and pink shrimp (*P. duorarum*). Each of these organisms was found to be pathogenic to shrimp when administered by intramuscular injection.*

INTRODUCTION

During 1972 and 1973, several large mortalities occurred in hatchery-reared brown shrimp (*Penaeus aztecus*) and white shrimp (*P. setiferus*) apparently as the result of a disease. A similar, if not identical disease, was observed in wild brown, white, and pink shrimp (*P. duorarum*) obtained from commercial bait dealers in Galveston. Mortalities from this disease ranged from a few shrimp lost per day in some cases to nearly 100 percent in other "die-offs." In these epizootics *Vibrio alginolyticus* was the organism most commonly isolated from diseased shrimp.

Vibrionic infections have been implicated as a major cause of mortality in juvenile penaeids in shrimp culture (Sindermann, 1971). *Vibrio parahaemolyticus*, the cause of an infectious food poisoning syndrome in Japan (Nickelson and Vanderzant, 1971) was isolated from white shrimp taken from Galveston Bay, Tex. (Vanderzant, Nickelson, and Parker, 1970). That same organism was found to be pathogenic to brown shrimp when bits of frozen white shrimp containing the organism were fed to brown shrimp, or when cultures of the organism were added to aquaria with brown shrimp.

V. parahaemolyticus has been isolated from diseased blue crabs (*Callinectes sapidus*) in Chesapeake Bay (Krantz et al., 1969; Colwell et al., 1972). Furthermore, *V. parahaemolyticus* or a very similar organism was isolated from Gulf of Mexico and South Atlantic coastal water and sediment samples (Ward, 1968), indicating that this organism which is potentially pathogenic to shrimp and other crustaceans is ubiquitous in many estuaries.

Lewis (In press) reported experiments in which adult brown shrimp were challenged with a field isolate of *V. anguillarum*. One-tenth ml of a 24-h broth culture diluted one hundredfold and introducted by injection beneath the dorsal carapace at the terminus of the rostral groove caused death of the shrimp within 5 days.

The microbial flora of brown and white shrimp from the Gulf of Mexico and from pond-reared brown shrimp have been studied (Vanderzant, Mroz, and Nickelson, 1970; Vanderzant et al., 1971). These studies indicated that the microbial flora of the Gulf shrimp and pond shrimp differ slightly. Bacterial counts of pond shrimp were reported to be much lower than those from Gulf shrimp (Vanderzant, Mroz, and Nickelson, 1970). The coryneforms (species of *Corynebacterium*, *Arthrobacter*, and *Microbacterium*) and to a lesser extent *Vibrio* were the predominant isolates from fresh pond-reared brown shrimp. In contrast, the microbial flora of Gulf shrimp was dominated by coryneforms and species of *Pseudomonas*, *Moraxella*, and *Micrococcus*.

This paper presents studies in which two *Vibrio* species were isolated from and found to be pathogenic to the penaeid shrimp, *Penaeus aztecus*, *P. setiferus*, and *P. duorarum*. Evidence is also presented that an *Aeromonas* sp. may cause a similar disease syndrome in these animals.

METHODS OF ISOLATION AND CULTURE

Bacteria were isolated from diseased laboratory-reared brown shrimp (*P. aztecus*) and white shrimp (*P. setiferus*) and from wild brown, white, and pink shrimp (*P. duorarum*) obtained by trawling from the Gulf or from local bait camps on West Galveston Bay. Juvenile and adult stages were represented. Cultures were obtained by extraction of hemolymphs by cardiac

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puncture in moribund shrimp over 40 mm in total length from the tip of rostrum to the tip of telson. In shrimp under 40 mm in total length, cultures were obtained by tissue impression of small pieces of abdominal muscle tissue onto the isolation medium. Shrimp that had been dead 1-2 h before necropsy were not considered suitable for bacterial isolation because of the advanced state of autolysis of the hepatopancreas and heart, and contamination of the hemolymph by enteric microorganisms.

The isolation media were tryptic soy agar (Difco Laboratories[1]) with 2 percent sodium chloride and tryptic soy agar with 7 percent NaCl. The inoculated media were incubated for 24-48 h at 28°C. Bacterial colonies were then transferred to fresh tryptic soy agar with 2 percent NaCl and streaked for individual colonies and incubated as described previously. This process was repeated until pure cultures were obtained of each organism present in the initial isolate.

All the bacteria studied were Kovac's oxidase positive, were motile by polar flagella, and initially required the presence of at least 2 percent NaCl in the medium for growth. None of the fermentative bacteria produced gas in glucose, lactose, sucrose, or mannitol. Those organisms which failed to produce lysine decarboxylase were beta hemolytic on 5 percent bovine blood agar and on the basis of their ability to produce arginine dihydrollase, 2-3 butanediol, gelatinase, and indole were identified as *Aeromonas* sp. (Eddy, 1960; Eddy and Carpenter, 1964; Schubert, 1967). Those organisms identified as *Vibrio* sp. were sensitive to 2, 4-diamino-6, 7- diisopropyl pteridine phosphate (Schubert, 1962), produced lysine and ornithine decarboxylase, indole, fermented sucrose, and grew in trypticase soy broth containing 10 percent NaCl. Further identification of the isolates was accomplished using methods described elsewhere (Lewis, 1973).

INFECTIVITY EXPERIMENTS

Heavily streaked 24- to 48-h cultures of each bacterial isolate were harvested in sterile saline (2 percent NaCl) and diluted to a standard concentration equal to a particulate suspension that allows 70 percent light transmission at a wavelength of 520-540 nm (Perkin-Elmer spectrophotometer model 124-Coleman). This standard suspension of bacteria contained approximately 10^7 bacterial cells/ml and was either administered directly to experimental shrimp or diluted by tenfold serial dilutions prior to being administered to shrimp.

Shrimp used in infectivity experiments were brown, white, and pink shrimp juveniles averaging 95 mm total length. The shrimp were obtained from laboratory-reared stocks or from local bait camps. Addition of bacterial isolates to the food given to the shrimp proved to be an unsuccessful means of infection. Therefore, inoculation by intramuscular injection was adopted as the most reliable method of ascertaining the pathogenicity of bacterial isolates.

Experimental shrimp were inoculated intramuscularly between the fifth and sixth abdominal segments with 0.05 ml of a bacterial suspension (approximately 10^5 bacterial cells) using a 1-cc tuberculin syringe. Control shrimp were given 0.05 ml sterile saline (2 percent NaCl) in the same location.

Experimental and control shrimp were maintained in 60-liter glass aquaria at 25-30°C for up to one week. Shrimp were checked twice daily for clinical signs of disease and mortality. Dead shrimp were removed.

CLINICAL SIGNS OF BACTERIAL DISEASE

The first apparent clinical sign of a lethal bacteremia was a gradual change from the usual colorless, translucent appearance of the musculature, particularly of the abdominal musculature, to a whitish-opaque coloration. Some animals examined also showed melanization of gill filaments, cuticular lesions, and ventrolateral edges of the carapace. A slight darkening of the dorsal portions of the integument (due to expansion of integumental melanophores) and a reddening of the pereiopods and the pleopods (due to expansion of integumental erythrophores) was usually apparent in moribund or freshly dead shrimp. Moribund shrimp commonly exhibited a pronounced dorsal flexure of the abdomen with the second and third abdominal segments at the apex of the flexure.

Behavioral signs of stress associated with the disease became more apparent as the disease progressed. These behavioral signs included reduced swimming activity, disorientation while swimming, and swimming on one side. Eventually, affected shrimp came to rest motionless on the bottom, some in an upright position supported by the pereiopods, pleopods, and telson, while others lay on their side. Some of these shrimp could be induced to brief periods of swimming activity by prodding. Death occurred usually 2-4 h after the shrimp had become lethargic. Occasionally shrimp remained in the upright position even after death.

Hemolymph drawn with a 1-cc tuberculin syringe directly from the heart of moribund shrimp typically would not clot as rapidly as hemolymph drawn from control shrimp. Hemolymph drawn in this manner from healthy shrimp clots very rapidly, often before it can be expelled from the syringe. The clotting of hemolymph from bacteremic shrimp is slower, requiring from 5 to 10 min after being drawn; hemolymph from some infected shrimp had not clotted 1 h after being drawn. Hemolymph drawn from most moribund shrimp having a bacteremia was slightly turbid in appearance and lacked the blue coloration that appears in clotted hemolymph of healthy shrimp. Giemsa stained hemolymph smears from moribund bacteremic shrimp contained hemocytes in greatly reduced numbers compared to normal shrimp. Gram stained hemolymph smears from the same animals showed the presence of numerous gram negative rods.

BACTERIA ISOLATED

Pure cultures of bacteria were usually obtained when hemolymph was drawn directly from the heart of moribund shrimp (Table 1). Cultures made from impression smears of small pieces of muscle tissue aseptically removed from the abdomen of small shrimp (under 40 mm total length) also frequently provided pure cultures of the presumed causative agent.

Vibrio alginolyticus and *V. anguillarum* were the most prevalent organisms isolated from shrimp with clinical signs of a bacteremia taken from

[1]Use of trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 1.—Organisms isolated and source of penaeid shrimp exhibiting clinical signs of a bacterial septicemia.

Date	Isolate number	Organism(s) isolated	Species[1]	Source, number of shrimp involved, and percent mortality
8/14/72	30	*Vibrio alginolyticus*	B (40 mm)	Lab-reared; 150; 20%
9/21/72	31	*V. alginolyticus*, *V. anguillarum*	W (100 mm)	Local bait camp; 50; 50%
1/9/73	53	*V. alginolyticus*	B (26 mm)	Lab-reared; 100,000; 99%
3/8/73	58	*V. alginolyticus*	B (58 mm)	Lab-reared; 200; 40%
3/16/73	59	*V. anguillarum*, *Aeromonas* sp.	B (90 mm)	Lab-reared; 10; 10%
3/18/73	61	*V. alginolyticus*	W,P,B (100 mm)	Local bait camp; 60; 50%
3/19/73	63	*V. alginolyticus*, *Aeromonas* sp.	B (90 mm)	Lab-reared; 10; 30%
3/20/73	64	*V. alginolyticus*	B (41 mm)	Lab-reared; 1,500; 99%
3/28/73	67	*V. alginolyticus*	B (120 mm)	Gulf of Mexico[2]; 7; 100%
4/18/73	73-74	*Vibrio* sp.	W,P (120 mm)	Local bait camp; 100; 10%

[1]B = brown shrimp (*Penaeus aztecus*), W = white shrimp (*P. setiferus*), and P = pink shrimp (*P. duorarum*). Average total length in parentheses.
[2]Held in laboratory tanks 40 days prior to onset of clinical disease.

Galveston Bay and vicinity and from hatchery-reared shrimp showing the same disease syndrome (Table 1). *Vibrio parahaemolyticus* was not isolated. An *Aeromonas* sp. was isolated along with *V. anguillarum* and *V. alginolyticus* from hatchery-reared shrimp from one location, but were not present in isolates taken from shrimp from other locations.

PATHOGENICITY EXPERIMENTS

Twenty-four to 48-h cultures of *V. alginolyticus*, *V. anguillarum*, and an *Aeromonas* sp. were found to be pathogenic to juvenile white, brown, and pink shrimp when the inoculum was administered directly to the shrimp by intramuscular injection in the fifth abdominal segment. The standard inoculum used to test the pathogenicity of bacterial isolates to healthy penaeid shrimp usually caused the death of exposed shrimp within 24-48 h. Most strains of *V. alginolyticus* caused the death of all shrimp tested within 24 h of inoculation (Table 2).

Injections of tenfold serial dilutions of the standard bacterial suspension resulted in progressively delayed times of appearance of clinical signs of disease and mortality (Table 2). Dilutions greater than 1:100 failed to produce clinical signs of disease or mortality in an experiment using a field isolate of *V. alginolyticus*.

DISCUSSION OF RESULTS

Every bacterial isolate obtained from obviously diseased penaeid shrimp was found to be pathogenic to healthy shrimp when administered directly by intramuscular injection. The virulence of the isolates varied, with some isolates causing 100 percent mortality in inoculated shrimp within 12 h while other isolates required considerably more time to kill the shrimp. Feeding of bacterial isolates to experimental shrimp seldom resulted in clinical disease. Other investigators have had similar difficulties in infectivity experiments. Lewis (In press) selected injection of *V. anguillarum* into experimental shrimp over other methods of exposure. Snieszko and Taylor (1947) were unable to infect American lobsters with *Gaffkya homari* introduced with the food, but succeeded in transmitting gaffkemia disease to healthy lobsters by injection of bacteria. It was later found that gaffkemia is transmitted only through ruptures in the integument and not through the consumption of infected food (Stewart and Rabin, 1970). This may also be the case in penaeid shrimp.

The presence of *Vibrio* species as part of the normal flora of pond-reared shrimp (Vanderzant et al., 1971) and the presence of these organisms in the water and sediments of estuaries (Ward, 1968) would tend to indicate that penaeid shrimp are resistant to infection by *Vibrio* by the oral route. Our experience has shown that handling of otherwise healthy hatchery-reared shrimp occasionally results in the onset of disease due, in most cases, to a *Vibrio* sp. The capture and holding in tanks of wild penaeid shrimp often result in occurrence of the same disease syndrome. Slight injuries resulting in interruption of the cuticle certainly occur when shrimp are subjected to rough handling or crowding in tanks. Cuticular injuries may provide a route of entrance for potentially pathogenic bacteria which are apparently a normal part of the microbial flora of shrimp (Vanderzant, Nickelson, and Parker, 1970; Vanderzant et al., 1971). Infection during ecdysis is also a distinct possibility.

Vibrio parahaemolyticus until now has been the most common *Vibrio* isolated from diseased crustacea. That organism is similar to *V. alginolyticus* but the latter differs from *V. parahaemolyticus* in its ability to swarm over the surface of the agar culture media within 12-24 h after inoculation at 28°C and in its ability to grow in media which does not contain salt.

The general gross appearance exhibited by moribund shrimp having a septicemic vibriosis has also been observed in moribund shrimp dying from causes other than a bacteremia. Gross clinical signs common to a bacteremia and to one or more other disease conditions include lethargy, areas of white discoloration of the abdominal musculature, a dorsal flexure of the abdomen, and redness of the pleopods and pereiopods. Shrimp exhibiting these signs and not having a bacteremia have been found to be suffering from hypoxia or anoxia due to the presence on the gills of large numbers of fouling organisms, the most

Table 2.—Mortality data from pathogenicity experiments in which brown, white, and pink shrimp were exposed to bacterial isolates obtained from diseased shrimp.

Species[1]	Mean length (mm)	Number tested	Organism	Accumulated mortality at hours post-inoculation				
				0	2	8	24	48
W	95	6	C-53, *Vibrio alginolyticus*	0			6	
B	150	1	C-53, *V. alginolyticus*	0			1	
W	95	6	Saline control	0			0	
W,P	96	14	C-58, *V. alginolyticus*	0	1	10	14	
W,P	95	5	C-53, *V. alginolyticus*	0	1	1	5	
W,B	92	5	C-58, *V. alginolyticus*	0	0	0	5	
W,P	84	5	C-59, *V. anguillarum*	0	0	0	4	5
W	95	5	C-63A, *V. alginolyticus*	0	0	4	5	
W	84	5	C-63B, *Aeromonas* sp.	0	0	3	5	
W,B	91	5	C-64A, *V. alginolyticus*	0	1	3	5	
W	81	5	C-64B, *V. alginolyticus*	0	0	3	5	
W,P	92	5	Saline control	0	0	0	0	0
W,P,B	95	5	C-53, *V. alginolyticus*	0		1	5	
W,P,B	95	5	1/10, C-53, *V. alginolyticus*	0	0	2	4	
W,P,B	95	5	1/100, C-53, *V. alginolyticus*	0		1	1	1
W,P,B	95	5	1/1,000, C-53, *V. alginolyticus*	0		0	0	0

[1]B = brown shrimp (*Penaeus aztecus*), W = white shrimp (*P. setiferus*), and P = pink shrimp (*P. duorarum*).

common of which is a stalked peritrich ciliate belonging to the genus *Zoothamnium*. Rigdon and Baxter (1970) described a similar condition in brown shrimp (*P. aztecus*) apparently due to anoxia with high temperature and handling stress as additive factors. These shrimp exhibited white discoloration and necrosis of the abdominal musculature. However, shrimp with a bacteremia can be distinguished from those suffering from anoxia or related conditions by the consistent presence of bacteria in the hemolymph.

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MFR PAPER 1144

A Comparative Study of the Bacterial Flora of the Hemolymph of *Callinectes sapidus*

R. R. COLWELL, T. C. WICKS, and H. S. TUBIASH

*ABSTRACT—The bacterial flora of blue crabs (*Callinectes sapidus*) from Chesapeake Bay was examined. Hemolymph of normal, healthy blue crabs was found non-sterile, with total bacterial counts ranging from <100 to >3.0 × 10^5 on a seawater-based medium and 0 to >10^5 on Standard Methods Agar. Counts of 0-10^3 were observed on TCBS agar.* Vibrio *spp., including* Vibrio parahaemolyticus, *were the major taxonomic groups found in the crab hemolymph. A comparison of crab data with that of Chesapeake Bay oysters revealed a qualitative difference in that the crab hemolymph flora was almost entirely* Vibrio *spp., whereas the oyster flora included species of a variety of other genera.*

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INTRODUCTION

The normal bacterial flora of blue crabs (*Callinectes sapidus*) from Chesapeake Bay has not been extensively studied. Interest in the microbiology of shellfish, traditionally, has focused on human pathogens found in commercially important species, as in the case of the blue crab. Many of the papers published on the microflora of the blue crab have concerned pathogens rather than the normal microbial flora present in the healthy animal (Couch, 1967; Fishbein et al., 1970; Krantz et al., 1969; Rosen, 1967; Sawyer, 1969; Williams-Walls, 1968). In addition, information has been gathered concerning the bacteriology of processing, handling, packaging, or storage of crabmeat (Loaharanu and Lopez, 1970; Ulmer, 1961; Ward and Tatro, 1970).

This study was conducted to determine the total viable, aerobic, heterotrophic, bacterial flora of blue crab hemolymph in freshly caught, aquarium-held, and market crabs during the summer months and to identify and classify the bacterial strains isolated, using the methods of numerical taxonomy. Numerical taxonomy as a method for the analysis of taxonomic data for bacteria was first proposed by Sneath (1957) and has been used, with modifications of the original methods, by various investigators (Society for General Microbiology, 1962; Beers et al., 1962; Silvestri et al., 1962; Sokal and Sneath, 1963). Colwell and Liston (1961), in one of the earliest numerical taxonomy studies, examined marine bacteria. Subsequently, several other investigators examined the taxonomy of marine bacteria (Floodgate and Hayes, 1963; Hansen et al., 1965; Liston et al., 1963; Pfister and Burkholder, 1965; Qualding and Colwell, 1964). Results of these studies proved helpful in identifying and classifying the bacterial flora from blue crab hemolymph.

MATERIALS AND METHODS

Healthy adult blue crabs (*Callinectes sapidus*) weighing 150-230 g were obtained from seafood markets or collected from several areas in Chesapeake Bay and Chincoteague Bay during the summer of 1970. Male and female crabs possessing hard carapaces ("soft-shell" crabs undergoing ecdysis were not included) were sampled to determine total numbers and types of aerobic, heterotrophic bacteria comprising their normal microflora during the summer months when the water temperature ranged between 23°-32°C. Sources of crabs, bacterial isolates, and dates of sampling are given in Table 1.

The isolates from the holding tanks were obtained from animals maintained in the laboratory for more than 1 week. Crabs obtained from the fish market were held in a tank for 1 to 2 days before sampling. Crabs from the Rhode River in Chesapeake Bay were sampled immediately after capture. Crabs from Marumsco Bar were also sampled in the field without delay after capture. The largest sample of animals examined was a catch made off Franklin City, Va. The hemolymph was sampled 12-16 h after capture.

Total viable counts of aerobic, heterotrophic bacteria in blue crab hemolymph were carried out as follows. After removing the fluid with a sterile 25-gauge needle and syringe wetted with a sterile solution of 5 percent sodium polyanethol sulfonate (GRO-BAX)[1,2], an anticoagulant reportedly effective for isolating bacteria from blood (Morello and Ellner, 1969), 1 ml of the hemolymph was withdrawn from each crab, either through the posterior hinge of the carapace or from the base of the coxa of the flippers. A dilution series was made, using a sterile diluent composed of four salts and distilled water in the concentration: NaCl, 24.0 g; KCl, 0.78 g; $MgCl_2$, 5.3 g; $MgSO_4 \cdot 7H_2O$, 7.0 g; and distilled water, 1 liter. One-tenth ml of each dilution was inoculated onto agar plates, in triplicate, and spread evenly with a sterile, bent glass rod. The three media used were: MSYE (Proteose peptone, 1 g; yeast extract, 1 g; agar, 15 g; four salts solution, as above; pH adjusted to 7.4-7.6 with 0.1 N NaOH), SMA, the Standard Methods Agar (Casein digest, 5.0 g; yeast extract, 2.5 glucose, 1.0 g; agar, 15 g; distilled water, 1 liter; pH adjusted to 7.0 with NaOH) and TCBS, the Thiosulfate-citrate-bile salts-sucrose agar[3]

[1]Roche Diagnostics, Hoffman-LaRoche, Nutley, N.J.
[2]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 1.—Source and date of isolation of bacterial strains included in the numerical taxonomy analysis.

Strain number	Date of isolation	Source[1]
1-10	7/15/70	HT
11-27	7/19/70	FM
28-33	7/21/70	RR
34-39	8/22/70	MB
40	8/27/70	FC
41	8/27/70	FC
42	8/27/70	FC
43	8/27/70	FC
44	8/27/70	FC
45	8/27/70	FC
46	8/27/70	FC
47	8/27/70	FC
48	8/27/70	FC
49	8/27/70	FC

[1]HT = Holding tank, Georgetown University; FM = Fish market, District of Columbia; MB = Marumsco Bar, Md. (Chesapeake Bay); RR = Rhode River, Md. (Chesapeake Bay); FC = Franklin City, Va. (Chincoteague Bay).

(yeast extract, 5.0 g; polypeptone peptone, 10 g; sodium citrate, 10.0 g; sodium thiosulfate, 10.0 g; oxgall, 5.0 g; sodium cholate, 3.0 g; sucrose, 20.0 g; NaCl, 10.0 g; iron citrate, 1.0 g; thymol blue, 0.04 g; bromthymol blue, 0.04 g; agar, 14.0 g; distilled water, 1 liter; pH 8.6). The inoculated media were incubated at 25°C for 48 h, after which counts were made and the media again incubated for an additional 5 days for a repeat count.

Forty-nine cultures were isolated, purified, and subjected to the testing procedure used for numerical taxonomy. Colonies were selected randomly and were streaked three times to ensure purity of the cultures. After purification, all cultures were tested for ability to grow on a medium (MSYE) containing the major ionic constituents of seawater. All cultures isolated from the SMA and TCBS media also grew on MSYE and were, therefore, maintained on MSYE.

Taxonomic tests were carried out on each pure culture for morphology and motility, using wet-mount preparations of 24-h broth cultures under phase contrast; gram stain; growth characteristics on MSYE agar after 48 h incubation at 25°C; growth at 4, 15, 25, 37, and 45°C; growth at NaCl concentrations of 0.0, 3.0, 7.0, and 10.0 percent; growth at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0; hemolysis of human blood; utilization of citrate; aerobic and anaerobic utilization of glucose and sucrose, employing MOF medium[4] (Leifson, 1963); utilization of galactose, mannitol, ribose, lactose, mannose; production of catalase, oxidase (Kovacs, 1956), indole, acetyl methyl carbinol, and urease; production of ammonia from peptone, NO_2, and NO_3; hydrolytic activity on starch, gelatin and casein; methyl red test; and utilization of alanine, proline, glutamic acid or methionine as sole carbon and nitrogen sources.

Drug sensitivity of each pure culture was determined using penicillin, 10 units; Chloromycetin, 30 μg; tetracycline, 30 μg; dihydrostreptomycin, 10 μg; and colimycin, 10 μg[5]. Sensitivity was recorded without quantification of the diameter of the inhibition zone. Sensitivity to the pteridine compound (0/129) was also recorded (Shewan et al., 1954).

All media, with the exception of the blood agar, were prepared with the salt solution, described above, as diluent. In most cases, dehydrated medium was mixed directly with the salt solution as diluent, but some media required special preparation. Media for the casein hydrolysis, methyl red, Voges-Proskauer, and carbohydrate utilization tests were prepared double strength in distilled water, and a double strength, sterile salt solution was added (1:1 vol/vol) after sterilization.

A total of 126 features used in the computer analysis were scored, with the code: 0 (negative), 1 (positive), or 3 (not tested or not applicable). The coded data were entered for analysis in an IBM 360/40 system equipped with discs and magnetic tape drives. Georgetown University Taxonomy Programs (GTP-1, 2, 4, and 5) were used in the analysis (Colwell, 1964; Colwell and Liston, 1961; Moffett and Colwell, 1968). The programs have been documented for the IBM Computer Users Library.

RESULTS

A wide range in bacterial counts for the blue crab hemolymph was observed. On the MSYE medium, counts ranged from <100/ml (five animals) to $>3.0 \times 10^5$/ml (five animals). Counts on SMA were lower, 0/ml (11 animals) to $>10^5$ ml (two animals), indicating that many of the bacteria found in the hemolymph require the major salts found in seawater for growth. On TCBS, the counts were from 0 (14 animals) to 10^3 (3 animals). The dominant colony type appearing on TCBS agar was yellow, 2 mm in diameter, and similar to *Vibrio alginolyticus*. Results of total viable counts are given in Table 2. No change in counts was observed between 48 h and 7 days.

Table 2.—Bacterial counts of the hemolymph of crabs collected at several locations on the media employed in the study. Counts given are per milliliter hemolymph of individual crabs. Figures given for each sample set are mean values, except where otherwise indicated.

Source	No. sampled	MYSE	SMA	TCBS
Laboratory holding tank	3	1.8×10^5	3×10^2	10
Fish market	2	6×10^4	4×10^3	1
Rhode River	6	2.1×10^5	60	0
Marumsco Bar	4	1.3×10^5	2×10^5	100
Franklin City, Va.	33	3.1×10^5	2×10^2	33
Total range	48	<100>3.0×10^5	0->10^5	0-10^3

The strain clusters obtained from the computer analysis of the taxonomic data are shown in Figure 1. All strains grouped at $S \geq 57$ percent and the major phenons detected are shown in Figure 1, A through I. Strains clustered in Phenon A were not found to be closely related, indicated by the low similarity values (62-64 percent). Phenon B consisted of strains more closely related to each other (S = 72-74 percent). Phenon C comprised the largest group, consisting of strains 41 through 11 (Figure 1) clustering at $S \geq 75$ percent. Three subgroups (I, II, III) were observed in Phenon C (Figure 1).

Phenon D, strain 9-24 (Figure 1) formed at S values $\geq$ 71-75 percent, indicating relatively high relationship among the strains. Phenon E formed at 72 percent S and was considered distinct from Phenon D. Phenons F and G formed at relatively low S values, 65-67 percent S and 68 percent S, respectively. Phenon H (intra S values of 62-63 percent) and Phenon I completed the groupings obtained in the analysis.

DISCUSSION

Counts on MSYE were comparable among sampling locations. Greater variations were noted for counts on SMA, with the lowest counts on SMA noted for Rhode River animals. Crabs from Marumsco Bar gave highest counts on SMA. The salinity of the water at Rhode River was lower than at Marumsco Bar and both the Rhode River and Marumsco Bar crabs were sampled immediately after capture. Hence, no correlation was noted between time of sampling after capture

[3]Baltimore Biological Laboratories, Baltimore, Md.
[4]Difco Laboratories, Detroit, Mich.
[5]Baltimore Biological Laboratory, Bioquest Division of Becton-Dickinson, Inc., Sensi-Disks.

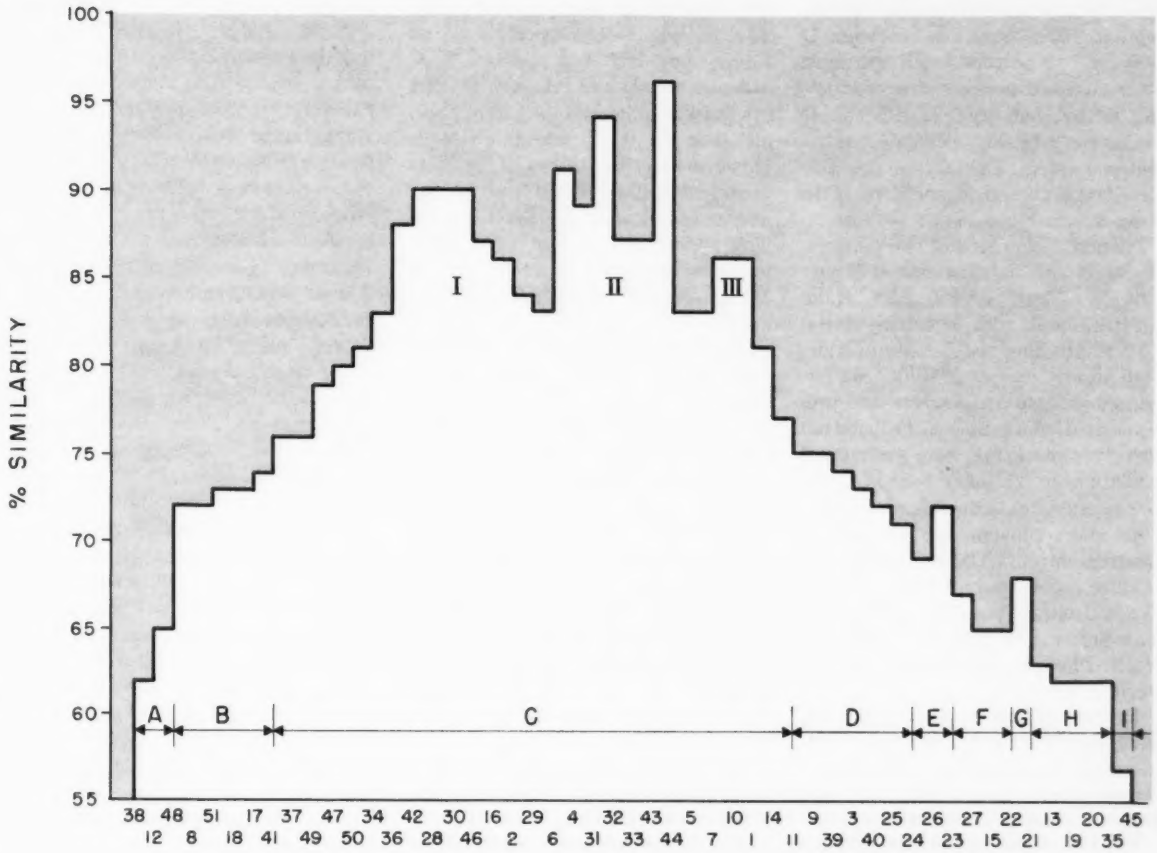

Figure 1.—Sorted output from the taxonomic analysis by computer. Groups were formed by highest linkage sorting.

and the salinity and the number of bacteria in crab hemolymph detected on SMA, a medium employed for detecting nonmarine bacteria. Also, no correlation between sex and total viable count on SMA was detected. However, it was clear that the proportion of nonmarine bacteria in crab hemolymph was higher for fish market and Marumsco Bar crabs. The holding tank and Franklin City samples exhibited approximately the same proportion of nonmarine bacteria (bacteria capable of growth on SMA).

The genera of bacteria found in crab hemolymph were obtained from the taxonomic data analyses, and each of the eight phenons were classified. Strains 38 and 12 (Phenon A) were gram-positive, motile rods, 1.3-3.0 μm long; capable of utilizing glucose and sucrose aerobically and anaerobically; growing at temperatures of 4°-37°C and pH 7.0-9.0; sensitive to penicillin, Chloromycetin, and colimycin; resistant to dihydrostreptomycin; being oxidase positive, indole negative, and citrate positive; utilizing alanine and proline as sole carbon and nitrogen sources; growing in 7-10 percent NaCl; and were classified as *Bacillus* sp.

Phenon B included five strains, one of which was the reference strain of *Vibrio parahaemolyticus* (strain Sak-3).

Phenon C was the largest group, with 27 strains comprising the cluster. All of the strains of Phenon C were round ended, motile, gram-negative rods, 1.3-3.0 μm long $\times$ 0.5-1.3 μm wide and occurring singly, not as pairs or chains. These strains grew at pH 4.0-9.0 and at temperatures of 15°-45°C, with half of the isolates capable of growth at 4°C and in 3-10 percent NaCl. However, 92 percent of the isolates were capable of growth without added NaCl. The strains formed convex, entire, translucent colonies yellow to white in color on MYSE agar. All fermented glucose anaerogenically and, in general, produced acid in sucrose, galactose, mannose, mannitol, and ribose and were lactose negative. All strains hydrolyzed starch, produced NH_3 from peptone, reduced nitrates, produced catalase, hydrolyzed casein, utilized citrate as a sole carbon source, were oxidase positive and methyl red positive. None of the strains of Phenon C produced urease or acetylmethylcarbinol, but half produced indole. All were sensitive to Chloromycetin; 64 percent were sensitive to dihydrostreptomycin; 36 percent to colimycin, and 16 percent to penicillin. Phenon C was classified as *Vibrio* spp.

Phenon D strains were straight, gram-negative rods, 1.3-3.0 μm long, occurring singly and in short chains (less than five cells). Colonies 2-5 mm in diameter and a translucent yellow or white were formed on agar. Glucose and sucrose were utilized aerobically and anaerobically, without gas. Acid was produced from mannose; nitrate

was reduced and NH_3 produced from peptone. All of the strains of Phenon D grew in 3-10 percent NaCl and most strains utilized citrate and were indole and methyl red negative. Phenon D strains were catalase positive and hydrolyzed casein. This phenon was also identified as comprising members of the genus *Vibrio*.

Phenon E consisted of two cultures, strains 23 and 26, clustering at 72 percent *S*. These strains were gram-positive, motile rods, occurring singly, 1.3-3.0 μm long × 0.7-1.5 μm wide. Both strains grew at 15°-45°C and fermented glucose and sucrose and produced acid from mannose. They did not hydrolyze casein and were oxidase and catalase negative. They were sensitive to penicillin, chloromycetin, tetracycline, and colimycin, but not to dihydrostreptomycin. The strains did not produce indole, were methyl red negative and citrate positive, did not produce urease, and grew in 7-10 percent NaCl. Phenon E was identified as a *Bacillus* sp.

The two strains, 15 and 27 (Phenon F) were identified as *Acinetobacter* sp. based on their inability to utilize the carbohydrates tested, their oxidase negativity, and catalase positivity.

The distinquishing features of Phenon G (strains 21 and 22) which clustered at 68 percent *S* were variable gram stain, rod shape and yellow colonies. The strains fermented glucose and sucrose, but did not reduce nitrates nor hydrolyze casein. They were oxidase positive, sensitive to dihydrostreptomycin, indole and methyl red negative, citrate positive, urease negative and grew in the presence of 0-10 percent NaCl. Phenon G was identified as a *Flavobacterium* sp.

Phenons H and I were not identified. They were motile, gram-negative rods which produced yellow to white colonies on MSYE agar at 15°-37°C and at pH 7.0-9.0. Strains in Phenons H and I fermented glucose and sucrose. The other characteristics tested varied among the strains of the two groups.

In summary, the isolates from the hemolymph of the blue crab belonged to the genera *Vibrio*, *Acinetobacter*, *Flavobacterium*, and *Bacillus*. The largest number of isolates, 38 of the 49 isolates examined, were identified as *Vibrio* spp.

A taxonomic distribution by source of the animals proved to be interesting. The holding tank isolates were all *Vibrio* spp., including phenons B, C (subgroups I, II and III), and D. The fish market isolates were *Vibrio* spp., including the B, C, and D phenons. However, representatives of the other genera were also isolated from the fish market samples. Rhode River isolates were all identified as *Vibrio* spp. (Phenon C, subgroups I and II). The Marumsco Bar isolates included phenons A, C, D, and H (*Vibrio*, *Bacillus*, and unidentified strains). The Franklin City isolates were predominantly of Phenon C (subgroups I and II) with some B and D phenons also observed to comprise the flora. Thus, the common feature of the crab hemolymph was the preponderance of *Vibrio* spp., particularly Phenon C vibrios which were present in crab hemolymph from all locations. A significant difference observed between the Marumsco Bar and fish market crabs and crabs from other locations in Chesapeake Bay was the presence of *Bacillus*, *Acinetobacter*, *Flavobacterium*, and other unidentified bacteria in the hemolymph.

The analysis was carried further in that the data for the 49 strains of this study were combined with data obtained from 181 other isolates from Chesapeake Bay blue crabs (unpublished data). The data for the 230 strains were subjected to computer analysis and the results showed 26 clusters at $S \geq 61$ percent with the largest cluster containing 68 strains. The 49 strains from the study reported here showed 22 strains clustered in the large, 68 strain phenon, identified as the genus *Vibrio*.

Thus, the results of the extended taxonomic analysis substantiates the observation that the predominant bacteria in the hemolymph of blue crabs are *Vibrio* spp.

An additional comparison was made of the data for the 49 crab isolates with data for 161 strains isolated from the Eastern oyster, *Crassostrea virginica*, which is found in Chesapeake Bay (unpublished data). The entire set of data for the 210 strains was analyzed by computer and it was found that the crab isolates, for the most part, did not cluster with and were distinct from the oyster strain clusters. Seven of the crab strains did cluster with oyster strains at $S \geq 61$ percent. These seven strains were largely of one phenon, Phenon D, members of which did not predominate crab hemolymph. The results suggest that the Phenon D *Vibrio* is more common in oysters than in crabs, with the phenon C *Vibrio* being more common in crabs. Further support for this observation can be drawn from the observations of Lovelace et al. (1968) who did a bacteriological analysis of oysters collected from the Marumsco Bar and Eastern Bay areas of Chesapeake Bay and found a predominance of *Vibrio*, *Pseudomonas*, *Achromobacter* spp., in that order, with a smaller percentage of *Corynebacterium*, *Cytophaga-Flavobacterium*, *Micrococcus-Bacillus*, and enteric species. *Pseudomonas*, *Achromobacter* and *Corynebacterium* spp. were not isolated from crab hemolymph in this study and only a few *Flavobacterium*, *Bacillus*, and other unidentified species were noted. However, the number of strains included in this study was relatively small. Nevertheless, the indication is that the composition of the microbial flora of the blue crab does not reflect geographical differences so strongly nor is so varied as that of the oyster (Lovelace et al., 1968).

In summary, the total number of viable, aerobic, heterotrophic bacteria found in hemolymph of healthy blue crabs is large and variable. Analysis of taxonomic data by computer revealed the predominant genus present in the hemolymph to be *Vibrio*, with members of *Bacillus*, *Acinetobacter* and *Flavobacterium* present in much smaller numbers. Clearly the hemolymph of most healthy blue crabs is not sterile.

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MFR PAPER 1145

Serum Changes in the Blue Crab, *Callinectes sapidus*, Associated With *Paramoeba perniciosa*, the Causative Agent of Gray Crab Disease

GILBERT B. PAULEY, MARTIN W. NEWMAN, and EDITH GOULD

ABSTRACT—Hemolymph from healthy blue crabs (Callinectes sapidus) *was compared with that of animals infected with* Paramoeba perniciosa, *the causative agent of gray crab disease. There was a significant difference (P = 0.01) between normal and gray crab sera in both the protein and the glucose values. Analysis of infected blue crab serum by both immunoelectrophoresis and acrylamide gel electrophoresis showed altered patterns of total protein and hemocyanin that could be accurately correlated with the severity of the disease.*

A previously unknown disease of the commercially important blue crab (*Callinectes sapidus*) which killed 20-30 percent of the animals held in commercial shedding tanks was described by Sprague and Beckett (1966). When viewed ventrally, moribund and dead specimens had a translucent gray appearance, hence the name "gray crab disease." Sprague and Beckett (1968) ascertained that the etiological agent of the disease was an amoeba, subsequently named *Paramoeba perniciosa*.

The amoebae cause lysis of muscle and blood cells and may completely replace the normal blood cells in the hemolymph prior to death of the crabs (Sprague et al., 1969; Sawyer et al., 1970). Epizootiological studies have shown the disease is ephemeral, occurring each summer in the latter part of June, and has a restricted range from Maryland to North Carolina (Sawyer, 1969; Newman and Ward, 1973).

In another commercially important crustacean, *Homarus americanus*, it has been found that bacteria, *Gaffkya homari*, cause serious alterations in hemolymph chemistry (Stewart et al., 1969; Stewart and Cornick, 1972). Therefore, a study was undertaken to determine whether the hemolymph from blue crabs infected with *P. perniciosa* differed from that of normal crabs. This paper reports our findings on the changes observed in the serum glucose, serum protein, and serum electrophoretic patterns in infected *C. sapidus*.

MATERIALS AND METHODS

The crabs, *C. sapidus*, used in this study were obtained from Chincoteague Bay, near Greenbackville, Va. Crab sampling began on 26 May 1972, with six subsequent samplings during June 1972. The sample size varied from 4 to 26 animals, with a total of 84 animals being examined. Control sera for immunoelectrophoresis were obtained from uninfected Chesapeake Bay crabs, taken near Oxford, Md.

The posterior portion of the carapace was swabbed with 70 percent ethanol before withdrawing hemolymph from the pericardial cavity with a sterile 5.0 or 10.0 ml syringe and 20-gauge 1½-inch needle. A drop of hemolymph from each animal was placed on a microscope slide and spread evenly in a thin film. This was allowed to air dry for 1-5 min and then placed in 10 percent neutral-buffered formalin seawater. Smears prepared from gray-appearing crabs were fixed in separate containers to avoid the possibility of amoebae floating off the slide and contaminating adjacent slides. Hemolymph smears were stained for 10 min with Giemsa[1] stain diluted 1:5 with phosphate buffer, pH 6.8, and examined for the presence of *P. perniciosa*. The slides were then scored as normal, light, moderate, or heavy infection, based on the relative number of amoebae present as described by Newman and Ward (1973). Hemolymph from healthy crabs, placed in a test tube, was allowed to clot around an applicator stick after which the clot was easily removed. Because the hemolymph of most crabs infected with *P. perniciosa* does not clot, it was centrifuged at 5,000 rpm for 10 min at 4°C, to remove cellular debris. The resultant sera were passed through Swinnex-25 millipore filters (0.45 μpore size) into sterile tubes, divided into 1.0 ml lots, dated, and frozen at −25°C. Sera from individual crabs were always kept separate.

Two rabbits were used for each antiserum produced. Freund's complete adjuvant (Difco) was mixed with an equal amount of crab serum and administered weekly for 5 weeks as 1.0 ml subcutaneous and 2.0 ml intramuscular injections in the rabbits. Two weeks after the final injection, antiserum was collected by cardiac puncture, filter-sterilized, and frozen at −25°C until used. Antisera were made against the sera from 13 infected crabs from Chincoteague Bay and from two normal male and two normal female crabs from Chesapeake Bay. Immunoelectrophoresis and staining were carried out as described by Pauley (1974).

The total protein concentration in the serum of 66 crabs was analyzed by the Folin-Ciocalteau method (Lowry et al., 1951). Serum glucose was determined for 60 crabs by the method of Hultman (1959). Because of the small sample size of some groups and because they were not homogeneous by analysis of the variance using the *F* distribution (Snedecor, 1956), no statistical tests for significance were attempted for the various groups other than between pooled

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

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normal and pooled heavily-infected gray crab samples. The *t*-test for comparing the means of two randomized groups of unequal size was used for statistical analysis of this data according to the procedure outlined by Snedecor (1956).

Aliquots (ca. 3 μl) of serum from individual blue crabs were subjected to electrophoresis at 4°C on 7 percent acrylamide gel columns, pH 9.1, with sample and stacker gels of 3 percent acrylamide, pH 5.2. Electrode buffer was 0.005M Tris (hydroxymethyl aminomethane) - 0.038 M Glycine, pH 8.3. Running time was 60 min at 1 milliampere/column followed by 70-75 min at 3 milliamperes/column with constant current. Both gel formulas and electrophoretic procedure are based on the work of Davis (1964) and have been fully described by Gould and Medler (1970).

Subsequent to electrophoresis, gels were stained for total protein or for copper. The stain used for total protein was Amido Schwartz 10 B (Buffalo Black), 1 percent in 7.5 percent acetic acid. Gels were destained by passive diffusion in several changes of methanol—glacial acetic acid—distilled water (5:1:5) for a total of about 20 h. Hemocyanin sites on the gels were marked by a stain for copper using an aqueous tetrazolium-cyanide solution (Gould and Karolus, In press), that proved to be faster and more consistently reliable than the classic rubeanic acid stain (Horn and Kerr, 1969).

BIOCHEMICAL ANALYSIS

Concentrations of total protein and glucose in the serum of heavily infected and uninfected crabs used in this study are listed in Table 1. The range, standard deviation, standard error, and mean of these samples are graphically presented in Figures 1 and 2. The distribution of the values was skewed in some groups toward the lower values, such as the glucose of normal females, where the value of the mean minus one standard deviation exceeded the lower limit of the range. As indicated in the Materials and Methods section, statistical tests of significance were performed only between pooled normal serum values and pooled heavily infected gray crab values. There was a significant difference ($P = 0.01$) in both protein and glucose values between the normal and the gray crabs. Serum concentrations of protein and glucose could often be correlated with the intensity of infection (Table 2).

Table 1.—Concentrations of total protein and glucose in normal and heavily parasitized blue crab serum.

	Total protein (mg/ml)					Glucose (mg/100 ml)				
	No.	Range	Mean	Std. dev.	Std. error	No.	Range	Mean	Std. dev.	Std. error
Normal										
Males	11	25.0-67.0	45.4	15.1	4.5	11	10.5-66.4	34.7	18.4	5.5
Females	11	5.3-22.4	14.6	5.6	1.7	15	7.9-74.4	24.4	18.6	4.8
Sponge	10	3.9-55.4	31.0	15.7	5.0	6	31.8-130.3	74.4	37.2	15.2
All	32	3.9-67.0	30.3	18.0	3.2	32	7.9-130.3	37.3	28.9	5.1
Gray										
Males	23	2.8-18.5	9.4	4.6	1.0	18	0-61.1	14.1	15.8	3.7
Females	11	2.8-13.7	7.4	3.9	1.2	9	0-29.2	9.4	10.4	3.5
All	34	2.8-18.5	8.8	4.4	0.8	28	0-61.1	11.1	14.1	2.7

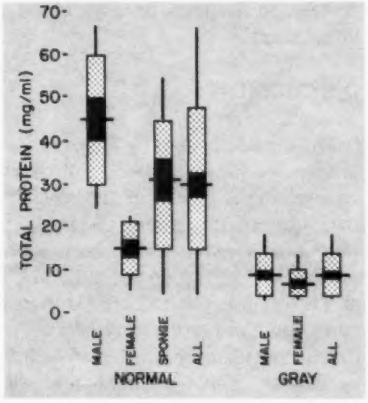

Figure 1.—Graphic presentation of the range, standard deviation, standard error, and mean of total serum protein concentration in normal and heavily parasitized blue crabs.

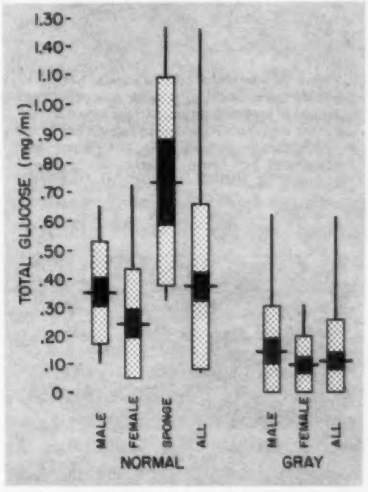

Figure 2.—Graphic presentation of the range, standard deviation, standard error, and mean of total serum glucose concentration in normal and heavily parasitized blue crabs.

IMMUNOELECTROPHORESIS

The normal immunoelectrophoretic patterns of male and female crabs were different (Figs. 3, 4). Infection of *C. sapidus* by *P. perniciosa* caused a progressive loss of serum protein as observed by immunoelectrophoresis, which was apparently related to the severity of the infection (Figs. 3, 4). There was some overlap in the appearance of the immunoelectrophoretic patterns of normal and lightly infected animals which were often not distinguishable (Fig. 5). Although no heavily infected female crabs were analyzed by this method, because of a shortage of hemolymph, there was never any doubt about a correct diagnosis with this method when hemolymph was tested from moderately or heavily infected male crabs.

ACRYLAMIDE GEL ELECTROPHORESIS

Typical electropherograms for male and female blue crabs with varying degrees of *P. perniciosa* infection show

Table 2.—Blood glucose, blood protein, and degree of infection by *Paramoeba perniciosa* in the exemplar blue crab hemolymph used for acrylamide gel pherograms.

Pherogram no. in figures 6 and 7	Date of capture	Sex	Blood glucose (mg/100 ml)	Blood protein (mg/ml)	State of infection observed in Giemsa stained blood smears
1	6/30/72	♀	—	2.8	Heavy infection
2	6/28/72	♀	2.5	6.5	Moderate infection
3	6/23/72	♀	3.9	17.5	Light infection
4	5/26/72	♀	37.1	43.9	Normal
5	6/28/72	♂	0.0	2.8	Heavy infection
6	6/30/72	♂	5.2	7.4	Moderate infection
7	6/16/72	♂	11.9	15.6	Light infection
8	6/9/72	♂	21.2	33.6	Normal

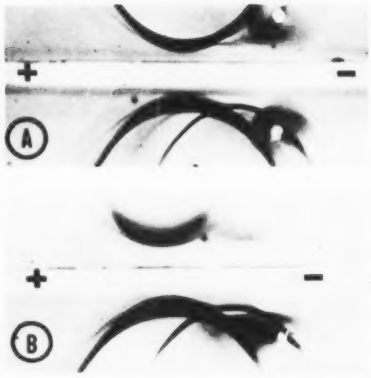

Figure 3.—Immunoelectrophoretic patterns of female blue crabs. A shows a lightly infected female at top and a normal female on the bottom. B shows a moderately infected female at top and a normal female on the bottom. Central trough contains rabbit antiserum against normal female crab serum.

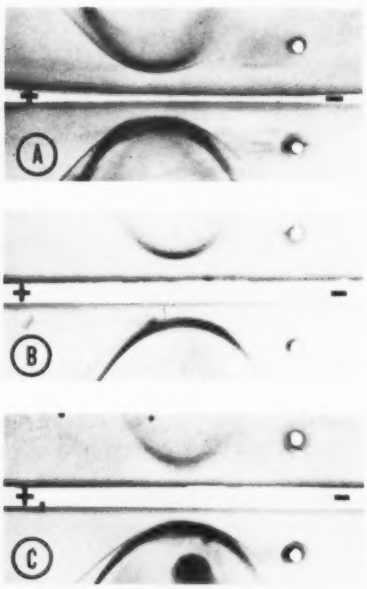

Figure 4.—Immunoelectrophoretic patterns of male blue crabs. A shows a lightly infected male at the top. B shows a moderately infected male at the top. C shows a heavily infected male at the top. Normal male patterns are on the bottom in all cases. Central trough contains rabbit antiserum against normal male crab serum.

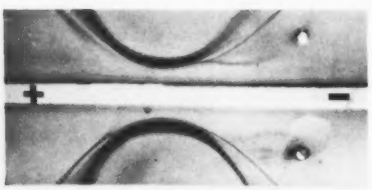

Figure 5.—Lightly infected male at top shows an immunoelectrophoretic pattern that is the same as the normal male at bottom. Rabbit antiserum against normal male crab serum is in central trough.

that both protein and serum copper decline (Figs. 6-9) in relation to the severity of the infection. The disappearance of the hemocyanin (HCy) copper (Figs. 6, 7) from the hemolymph of infected crabs is more rapid than the disappearance of the protein moiety (Figs. 8-10), a circumstance probably reflecting the relative proportions of copper and protein in the HCy molecule. The two fast HCy's ($\alpha -1$ and $\alpha -2$) in the normal examples, Figures 6 and 7, are the last proteins to disappear from the blood of infected crabs.

DISCUSSION

The disease caused by *P. perniciosa* is systemic (Sawyer, 1969) and effects changes in the blue crab that resemble the alterations seen in lobsters (*Homarus americanus*) infected by the bacterium *Gaffkya homari*. Sawyer et al. (1970) observed a decline of hemocytes and a reduced ability of crab serum to coagulate when infected with *P. perniciosa*. These changes have also been observed in infected lobsters (Stewart et al., 1969; Stewart and Rabin, 1970). Diet alone is capable of influencing crustacean hemocyte numbers (Stewart et al., 1967), and the presence of actively phagocytizing cells in invertebrates does not insure pathogen destruction (Cornick and Stewart, 1968; Pauley et al., 1971). However, in the case of *P. perniciosa* in blue crabs, these changes probably demonstrate the pathogen's ability to overcome two of the most important defense mechanisms of crustaceans: phagocytosis and coagulation (Bang, 1970; Sindermann, 1971).

The normal glucose values obtained in this study for *C. sapidus* (7.9-130.3 mg/100 ml, $\bar{x}$ = 37.0 mg/100 ml) are much lower and show a greater range than those listed by Florkin (1960) for this species. However, Lynch and Webb (1973b) have found a considerable variation in the serum glucose level of blue crabs throughout the year. It is known that lack of food will cause significant hypoglycemia in *C. sapidus*, but this stress factor alone is not capable of reducing the glucose level to zero (Florkin, 1960), a value observed in several diseased blue crabs in this study. Stewart and Cornick (1972) found a virtual disappearance of glucose from the hemolymph and a reduction in total carbohydrates in *H. americanus* infected with *G. homari*. The reduction of glucose which we observed in *C. sapidus* may be attributed, at least in part, to uptake and utilization by *P. perniciosa*, resulting from the hosts' inability to compete successfully for their own nutrients, as in the case in lobsters infected with *G. homari*.

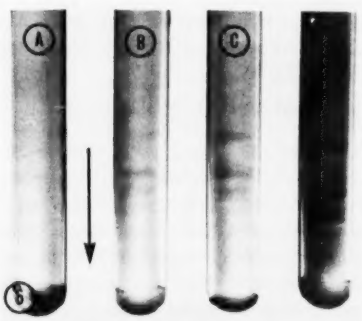

Figure 6.—Pherogram of female crab hemolymph stained for copper. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

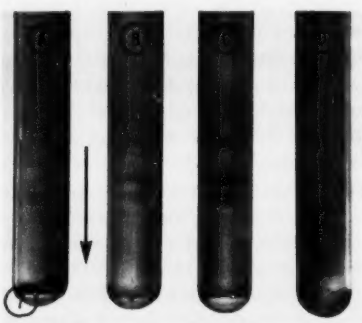

Figure 7.—Pherogram of male crab hemolymph stained for copper. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

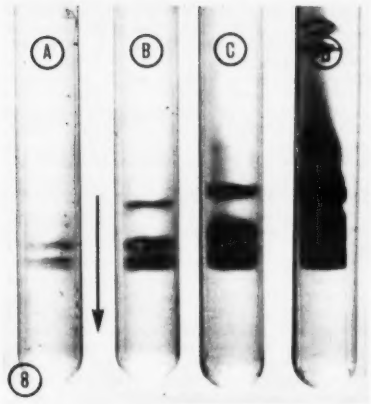

Figure 8.—Pherogram of female crab hemolymph stained for total protein. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

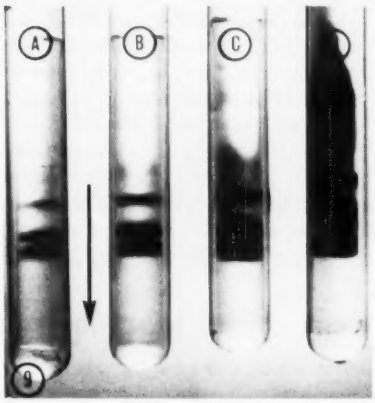

Figure 9.—Pherogram of male crab hemolymph stained for total protein. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

Normal total protein values obtained in this study for *C. sapidus* serum (3.9-67.0 mg/ml, x = 30.3 mg/ml) are in close agreement with the normal values observed in lobsters (Stewart et al., 1967) but are somewhat lower than those values observed in the blue crab by other investigators (Lynch and Webb, 1973a). In lobsters infected with *G. homari*, there is a significant, but not drastic, decline in serum protein, which is not regarded as a serious factor in the deterioration of the animals' physiological condition (Stewart et al., 1969). In blue crabs, the drop from 30.3 mg/ml to 8.8 mg/ml in infected animals is significant and probably accounts, in part, for the extremely weakened condition and rapid deterioration of crabs infected with *P. perniciosa*. Stewart et al. (1967) have shown that protracted diet changes can cause severe serum protein losses in the American lobster. However, in the infected blue crabs, diet change alone probably would not account for the large drop in serum protein, because the diseased crabs are captured in baited crab pots within 24 h of examination, indicating that they feed actively into the final stages of the disease. The amoebae are probably either pinocytosing serum proteins of the crab or secreting proteolytic enzymes which hydrolyze them into small peptides and amino acids, which may then be consumed by the parasite. The loss of fibrinogen as part of the total serum protein by either of these methods would account for the failure of the infected crab hemolymph to clot.

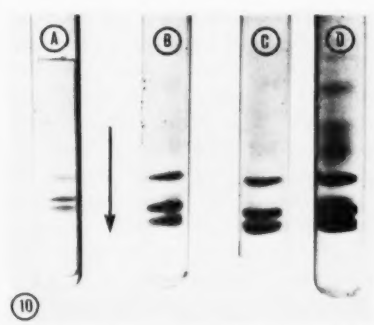

Figure 10.—Pherogram of male crab hemolymph stained for total protein. Hemolymph samples are from the same three infected animals whose immunoelectrophoretic patterns are shown in Figure 4. Heavy infection - A; moderate infection - B; light infection - C; normal - D. Arrow indicates migration direction.

The secretion of powerful enzymes by the parasite would also account for the extensive cellular lysis observed in diseased animals. The rapid destruction of the slow HCy (β) during infection by *P. perniciosa* does not occur with lyotrophic agents, such as urea, in which case it is the fast HCy (α) that are destroyed initially (Gould and Karolus, In press). This is further evidence that the amoebae, directly or indirectly, are altering covalent bonds of molecules within the host. Since hemocyanin is the oxygen binding and transporting molecule of crustaceans, the loss of hemocyanin indicates there probably is insufficient oxygen binding and transport. Death, therefore, may be due to a combination of insufficient oxygen and nutrients.

Minor electrophoretic changes were observed in infected lobsters (Stewart et al., 1969), but these irregularities were not greatly pronounced at the time of death. Analysis of infected blue crab serum by both immunoelectrophoresis and acrylamide gel electrophoresis showed significantly altered patterns of total protein and hemocyanin that could be correlated with the severity of the disease in all but 4 of the 79 cases analyzed. Acrylamide gel protein patterns corroborate the results obtained with immunoelectrophoresis (Fig. 10). Copper electropherograms may also be sensitive indicators of infection because of the rapid disappearance of hemocyanin during the disease. Although electrophoretic analysis is probably not specific for this infection, we recommend acrylamide gel electrophoresis of the serum to determine the degree of stress because of its relative ease of performance and clarity of results.

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MFR PAPER 1146

Microsporidian Infections of Amphipods with Special Reference to Host-Parasite Relationships: A Review

H.-P. BULNHEIM

ABSTRACT—A survey of the 10 species of microsporidians associated with amphipod crustaceans is presented. Specific features for their recognition are given on taxonomy, morphology, spore size, sites of infection, and host range. Some aspects of the host-parasite relationships are considered. In several species, transovarian transfer appears to be a major route of transmission. Previous results on microsporidians indicate that they do not cause fatal diseases in their amphipod hosts. A sex-determining influence on the progeny of Gammarus duebeni *after infection by* Octosporea effeminans *or* Thelohania herediteria *is indicated. Both of these microsporidians are transmitted through the eggs to the host's offspring which, in general, all develop into females. Some results and problems of this effect on sex ratio are discussed.*

INTRODUCTION

Microsporida are cytozoic parasites with a wide host range, comprising all phyla of the animal kingdom from protozoans to mammals. They are important pathogens of arthropods, especially insects, since they cause severe diseases in several members of this group. Compared with the numerous detailed investigations of microsporidioses in insects, very few studies are devoted to microsporidian infections in crustaceans, and to the effects they have on their hosts. Since Kudo's review (1924), no comprehensive and critical report on the taxonomy and biology of the microsporidians detected in crustaceans has been published, except for a survey recently presented by Sprague (1970a) on protozoan parasites in decapod Crustacea. Sprague recorded less than two dozen microsporidian species, mainly from marine hosts, with some known to cause diseases in commercially important decapods.

Due to several inadequate descriptions and some taxonomic confusion, it is difficult to estimate the exact number of microsporidian species occurring in crustaceans. Moreover, the widely scattered literature supplies little or no information on host-parasite relationships. Therefore, only scanty data are available for an assessment of their pathological significance.

This paper is confined to the microsporidian species parasitic in amphipod crustaceans, and presents a survey of their taxonomy, specific morphological characters, sites of infection, and host range. In addition, various aspects of the host-parasite relationships are considered.

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TAXONOMIC SURVEY

Microsporidia are protozoan parasites which are characterized by the formation of spores equipped with an extrusible polar filament. Their life cycle commences with the liberation of a uninucleate or bi-nucleate sporoplasm from the spore with subsequent entry into a host cell. An intracellular multiplicative phase (schizogony) is followed by the development of spores (sporogony). The resistant spores infect new hosts.

Among the criteria employed for the separation and identification of microsporidian species, the most significant characters are shape and size of the spores. Besides the morphometrics of the spores, the various stages of the life cycle, the mode of sporogony, the sites of infection, and the host specificity are of diagnostic importance. However, since some original descriptions are vague and fail to provide adequate detail, a satisfactory review of all species recorded in amphipods cannot be presented. Nevertheless, an attempt has been made to list all species described and to summarize specific features for their recognition (Table 1). The list is based on a survey presented by Lipa (1967), but includes several additions and corrections. In several species the dimensions of fresh spores are given, but in others it is doubtful whether size refers to fixed or fresh spores.

The microsporidians recorded from amphipods belong to 10 species from 5 genera. Most host species belong to the genus *Gammarus* which occurs in freshwater as well as brackish and marine habitats. Some additional data for characterization of the microsporidian genera (cf. Weiser, 1961) and species are briefly presented below.

Genus *Nosema* Nageli, 1857

The essential characteristic of this genus is that only one spore is produced by each sporont.

Nosema gammari van Ryckeghem has been described from only one specimen of *Gammarus pulex*, found in Belgium (van Ryckeghem, 1930). A diffuse infection of the host muscles was observed, but no further details on the life history have been reported for this parasite. In England, Pixell-Goodrich (1929, 1956) noted *Nosema* sp. in *G. pulex*, but no detailed characteristics of this species have been published.

Nosema kozhovi Lipa was recorded (Lipa, 1967) in *Brandtia lata lata* collected on the shores of Lake Baikal (Soviet Union). All tissues were infected with especially heavy concentrations in the gut epithelium, adipose tissue, and muscles. Parasitized animals had a milky-white appearance.

Genus *Thelohania* Henneguy, 1892

This genus develops eight spores from a single pansporoblast and the spores may remain together within a common membrane.

Table 1.—List of microsporidian species parasitic in amphipod crustaceans.

Microsporidian species	Host species	Sites of infection	Size of spores (μm)	References
Nosema gammari van Ryckeghem	*Gammarus pulex* L.	peri- and intermuscular tissue	1.5 by 0.75	van Ryckeghem (1930)
Nosema kozhovi Lipa	*Brandtia lata lata* (Dybowski)	gut epithelium, adipose tissue, muscles	3.3-3.9 by 2.1-2.2 (fresh)	Lipa (1967)
Thelohania mülleri Stempell	*Gammarus pulex* L., *Gammarus chevreuxi* Sexton	muscles	4-5 by 2 (fresh)	Pfeiffer (1895), Stempell (1902), Léger and Hesse (1917), Jírovec (1936), Pixell Goodrich (1929, 1956), Bulnheim (1971a)
Thelohania mülleri var. *minuta* van Ryckeghem	*Gammarus pulex* L.	muscles	3 by 1.5	van Rycheghem (1930)
Thelohania vandeli Poisson	*Niphargus stygius* Schiödte	muscles (?)	6-6.5 by max. 3	Poisson (1924)
Thelohania herediteria Bulnheim	*Gammarus duebeni* Lilljeborg	muscles, ovaries	4.5-6 by 1.9-2.7 (fresh)	Bulnheim (1969, 1971a)
Stempellia mülleri (Pfeiffer) [syn.: *Glugea mülleri* Pfeiffer (partim), *Plistophora mülleri* (Pfeiffer), *Thelohania giraudi* Léger and Hesse, *Plistophora blochmanni* Zwölfer]	*Gammarus pulex* L., *Gammarus locusta* (L.), *Gammarus oceanicus* Segerstråle, *Gammarus salinus* Spooner, *Gammarus zaddachi* Sexton, *Gammarus duebeni* Lilljeborg, *Niphargus ilidżensis* Schäfer	abdominal muscles	4-5.5 by 2.5-3 (fresh)	Pfeiffer (1895), Labbé (1899), Stempell (1902), Léger and Hesse (1917), Debaisieux (1919, 1928), Zwölfer (1926a, 1926b), Géorgévitch (1929), van Ryckeghem (1930), Bulnheim (1971b)
Octosporea gammari van Ryckeghem	*Gammarus pulex* L.	excretory system, heart epithelium	4-6 by 1.2-2	van Ryckeghem (1930), Jírovec (1943)
Octosporea effeminans Bulnheim and Vávra	*Gammarus duebeni* Lilljeborg	ovarial tissue, adipose tissue	4-10 by 1.5-2.5 (fresh)	Bulnheim (1967, 1969, 1970), Bulnheim and Vávra (1968)
Bacillidium niphargi (syn.: *Mrazekia niphargi* Poisson)	*Niphargus stygius* Schiödte	?	8-9 by 2	Poisson (1924), Jírovec (1936)

Thelohania mulleri Stempell, a parasite of *G. pulex*, was described in detail by Stempell (1902). Evidently the same microsporidian was found in *Gammarus chevreuxi* by Pixell-Goodrich (1929). The papers of Léger and Hesse (1917), van Ryckeghem (1930), Jírovec (1936) and Bulnheim (1971a) also deal with this species. Initially, *T. mulleri* was confused with *Stempellia mulleri*, as outlined in a subsequent paragraph. The infected host displays a spotted, whitish, opaque coloring. Both the body muscles and the muscles of the appendages become heavily infected. The spores are typically pyriform in shape, but anomalies of spore formation occasionally may occur with the development of four or even two spores that are significantly larger than the others within a pansporoblast. Host records derive from Germany, England, and Belgium.

T. mulleri var. *minuta* van Ryckeghem is a smaller microsporidian than *S. mulleri*, with the same characteristics of spores and sites of infection (van Ryckeghem, 1930). Without re-examining both forms, it is difficult to decide whether they represent distinct species.

Thelohania herediteria Bulnheim is clearly distingishable from *T. mulleri* by its larger size and more cylindrical spores (Fig. 1). It invades the muscles and ovaries of female *Gammarus duebeni*, but does not occur in males (Bulnheim, 1971a). Although the posterior part of the body is more heavily parasitized than the anterior, infection of the host cannot be recognized macroscopically. The only record of this species is from the River Elbe estuary in Germany.

Genus *Stempellia* Leger & Hesse, 1922

In this genus, 4, 8, 16, or 32 spores develop within one pansporoblast.

Stempellia mulleri (Pfeiffer) has a rather complicated taxonomic history. Pfeiffer (1895) described a species from *G. pulex* named *Glugea mulleri*, which is similar to *S. mulleri*. Later on it was named *Plistophora mulleri* (Pfeiffer) by Labbé (1899) and *T. mulleri* (Pfeiffer) by Stempell (1902). Debaisieux (1919) and Zwölfer (1926a) stated that the form discern. One species is to be regarded as *T. mulleri*, the other as *S. mulleri*. The latter appears to be identical with the descriptions of *Thelohania giraudi* (Léger and Hesse, 1917), *G. mulleri* descriptions of *Thelohania giraudi* (Léger and Hesse, 1971), *G. mulleri* (Debaisieux, 1919, 1928) and *Plistophora blochmanni* (Zwölfer, 1926a, 1926b). Géorgévitch (1929) recognized this synonymy and named the microsporidian in question *P. mulleri* (Pfeiffer). According to the numbers of spores produced within one pansporoblast, it must be transferred to the genus *Stempellia*. The spores have an ovoid shape (Fig. 2). *S. mulleri* has a wide host range as demonstrated by several infection experiments (Bulnheim, 1971b). Therefore, it can be expected that this parasite may invade amphipods other than those listed in Table 1. Host records derive from Germany (freshwater and euryhaline species), Belgium, and Yugoslavia. *S. mulleri* can be easily recognized by the presence of one or more white tubular masses of parasites which are arranged in the abdominal muscles of its hosts and separated from one another by 2-3 mm.

Genus *Octosporea* Flu, 1911

Species of the genus *Octosporea* have cylindrical spores which may be slightly arched. The spore width is one-third or less of the spore length, and all stages of the life cycle are binucleate.

Octosporea gammari van Ryckeghem was detected in one specimen of *G. pulex* from the region of Louvain in Belgium. Van Ryckeghem (1930) noticed in this specimen a dual infection of *O. gammari* and *T. mulleri*. Jírovec (1943) later observed the same microsporidian near Prague, Czechoslovakia. It invades the excretory system and the tissue around the heart.

Octosporea effemininans Bulnheim and Vávra (Fig. 3) has been found in the

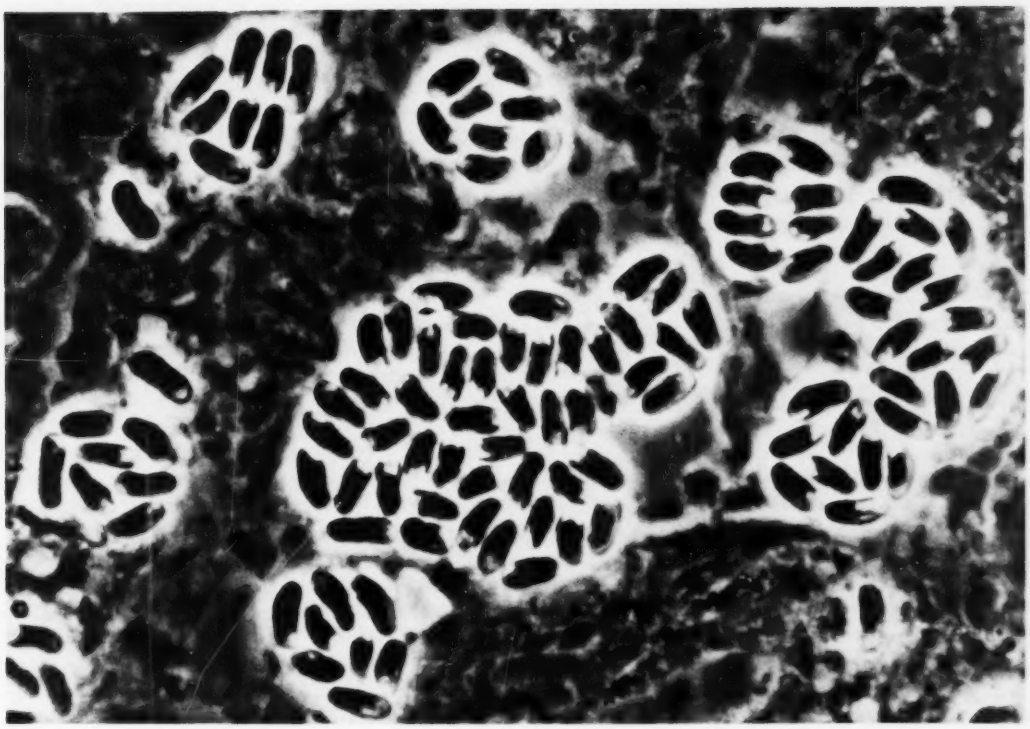

Figure 1.—*Thelohania hereditaria*. Fresh spores from infected *Gammarus duebeni*. (Phase contrast, 2,000×).

ovaries and adipose tissue of the brackish-water species *G. duebeni* (Bulnheim and Vávra, 1968). This parasite occurs only in females and, sometimes, in intersexes. This parasite was found in populations of *G. duebeni* from the River Elbe estuary in Germany and the Baltic Sea. Dual infections by this species and by *T. herediteria* have been observed in a few cases (Bulnheim, 1971b).

Genus *Bacillidium* Janda, 1928

The genus *Bacillidium* is characterized by very long, rod-shaped spores. In contrast to members of the genus *Mrazekia*, they have no tails (Weiser, 1961). However, Sprague (1970b) pointed out that the genus *Bacillidium* was falsely distinguished from *Mrazekia* by comparison with a species incorrectly regarded as the type of the latter genus and is a junior subjective synonym of *Mrazekia*.

Bacillidium niphargi (Poisson) has been reported to occur in the subterranean amphipod *Niphargus stygius*. It was first named *Mrazekia niphargi* by Poisson (1924), but the spores did not possess tails. Jírovec (1936), therefore, assigned this microsporidian to the genus *Bacillidium* and named it *B. niphargi*. Unfortunately, no further biological data have been recorded on this parasite.

Unnamed Species

In the muscles and heart of *Talitrus* sp., Mercier (1906) observed a microsporidian. Anders (1957) detected a microsporidian, whose taxonomic position could not be clearly identified, associated with *G. pulex subterraneus*. He found this parasite in intersexual specimens. Recent studies by the author have demonstrated that *Gammarus locusta* and *Gammarus salinus* may also be attacked by *Thelohania* sp. which have smaller spores than *T. herediteria* but similar morphology and sites of infection. Whether or not they represent a new species requires careful investigation and comparison with the known species of the genus *Thelohania*.

From these observations it is obvious that a considerable diversity of microsporidian species exists in amphipods, and that many new microsporidian species will be discovered in this crustacean group.

TRANSMISSION

In general, the infection of a susceptible host is initiated by the ingestion of spores. It is assumed that the digestive fluids then cause the extrusion of the polar filament and the discharge of the sporoplasm which penetrates a host cell. Transovarian transfer represents a route of infection which is probably the major method of transmission of microsporidians in amphipods studied thus far.

An experimental infection of laboratory-reared gammarids by uptake of spores with food was achieved in *S. mulleri* (Bulnheim, 1971b). Although Zwölfer (1926a) failed to infect *G. pulex* in this way, Bulnheim (1971b) transmitted this species by feeding starved gammarids with infected musculature from other specimens to demonstrate the wide range of possible hosts for this microsporidian.

In contrast to *S. mulleri*, which does not invade the ovaries, several other species have been shown to be transferred through the eggs of their hosts.

This mode of transmission was recorded in *O. effeminans* and *T. herediteria* (Bulnheim and Vávra, 1968; Bulnheim, 1971a). Further investigations and reexamination provided evidence that transovarian transfer also occurred in *Thelohania* sp. associated with other representatives of the genus *Gammarus*: *G. locusta*, *G. salinus*, and *G. pulex*. However, the taxonomic position of *Thelohania* sp. from the latter amphipods could not be clarified, but the microsporidian found in *G. pulex* was assigned to *T. mulleri* var. *minuta*, which was briefly described by van Ryckeghem (1930).

In the microsporidians transferred transovarially, infection of the oocytes and multiplication by schizogony is intimately associated with the reproductive cycle of the female hosts. Their ovaries are long paired tubes which contain oogonia and three layers of different-sized oocytes. Generally, the oocytes are not infected before they are arranged in the third layer in order to undergo intensive cytoplasmic growth. As a rule, only vegetative stages, mainly binucleate schizonts, can be found in the oocytes. The propagation of the parasites within the ooplasm proceeds until the formation of yolk granules takes place. When the eggs are released into the brood pouch and fertilized, the parasites undergo a second period of schizogonic multiplication which leads to a strong infiltration of the developing embryo by vegetative stages.

Attempts to achieve peroral transfer of the microsporidians *O. effeminans* and *T. herediteria* to their host *G. duebeni* did not succeed. Although fresh and dried tissues contaminated by spores were offered to the test animals, an experimental infection could not be accomplished. The reasons for this failure are not clear, but may be due to the fact that extrusion of the filament from the spore cannot be instigated by chemical or mechanical stimuli in these species. Nevertheless, a natural infection by uptake of spores scattered in the aquatic environment cannot be excluded. However, transovarian transfer of vegetative stages appears to be the most important and effective way of regularly transmitting an infection.

PATHOGENICITY

Epizootic diseases such as microsporidian infections may cause various histopathological alterations, often resulting in an enormous hypertrophy. However, no evidence of such pronounced pathological changes from microsporidian infections in amphipods has been provided. As a result of progressive multiplication of the parasites, the muscles become filled up with ripe spores, ultimately destroying the host muscle fibers. In some gammerids infected with members of the genera *Nosema* and *Thelohania*, Pixell-Goodrich (1929) observed yellow or brownish chitinoid nodules which constituted necrotic muscle fibers that were detached from the surrounding tissues. These muscles were then attacked by phagocytes which also ingested spores. Owing to the secretion of chitinoid substance by these hemocytes, the areas where the attack was most advanced exhibited the darkest brown color.

A similar defense mechanism of the host appears to occur in *G. pulex* infected by *S. mulleri*. The large masses

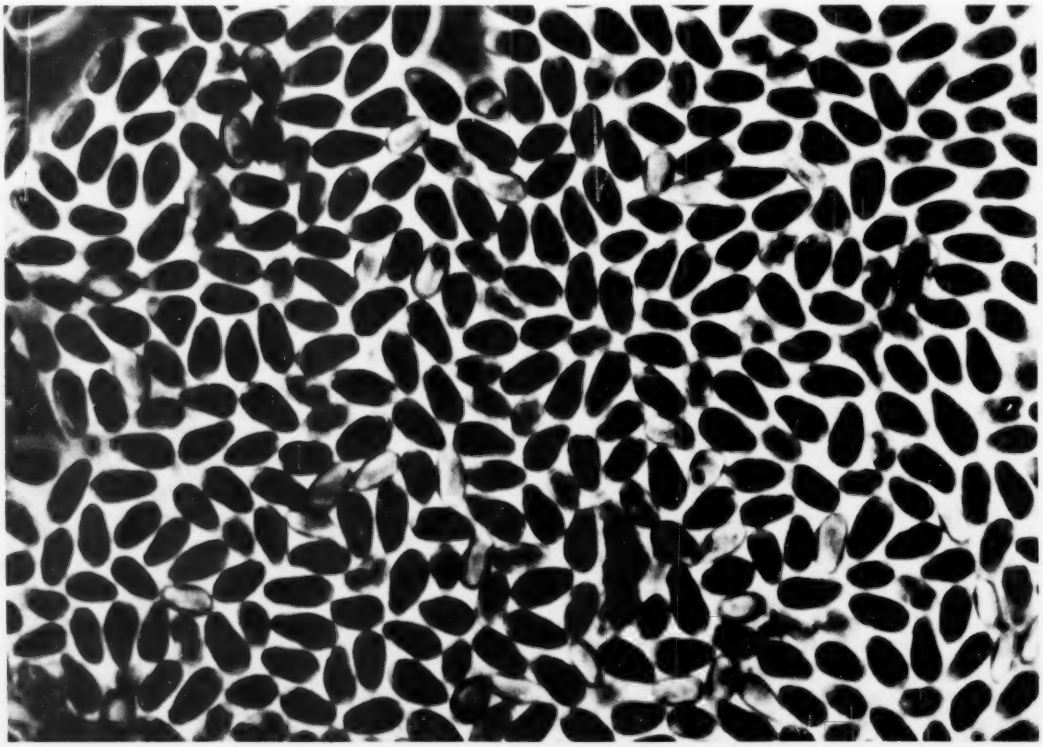

Figure 2.—*Stempellia mulleri*. Fresh spores from infected *Gammarus salinus*, some with extruded sporoplasm. (Phase contrast, 2,000×).

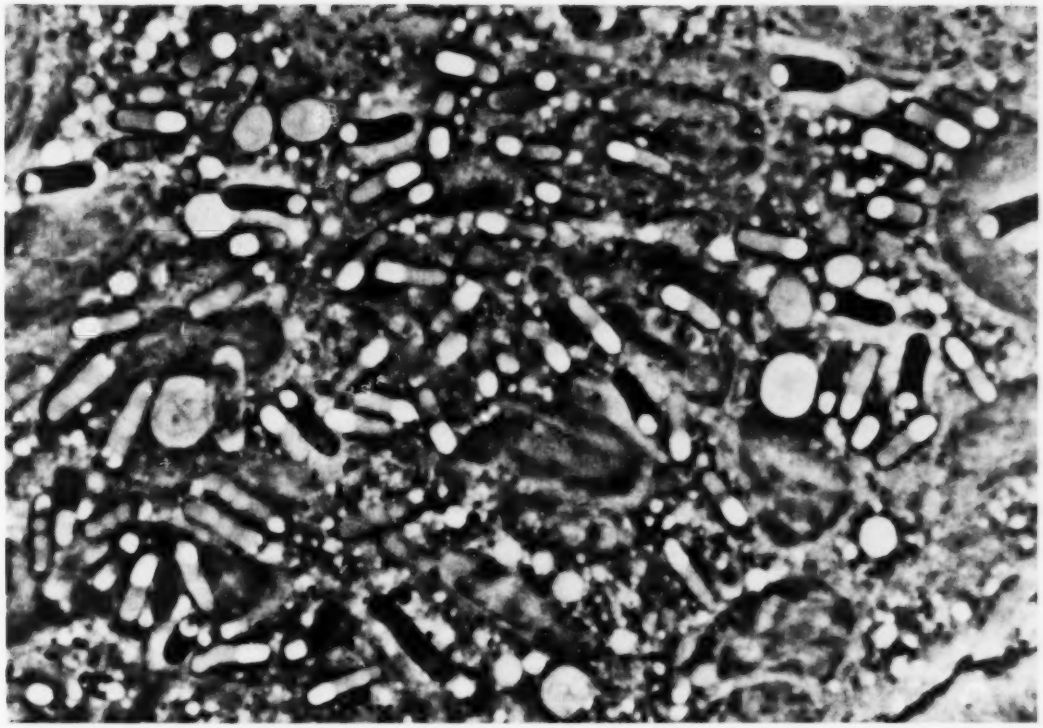

Figure 3.—*Octospora effeminans*. Fresh spores and sporonts together with a binucleate schizont (left) from infected *Gammarus duebeni*. (Phase contrast, 2,000×).

of parasites distributed within the abdominal muscles are sometimes surrounded by a brownish envelope, as observed by Zwölfer (1926a). He also noticed partially digested spores and sporoblasts at the periphery of such zones and suggested that the connective tissue of the host might be responsible for this encapsulation. Only severe infections, which lead to a complete breakdown of the muscles, can cause this reaction by the host (Zwölfer, 1926a). Similar responses in tissues invaded other than muscles have not been reported.

With respect to the effects of the parasites on their hosts, some findings have been recorded which indicate that none of the microsporidian species which infect amphipods cause fatal diseases, and thus do not seriously affect the life functions of their hosts. Lipa (1967) stated that specimens of *B. lata lata* infected by *N. kozhovi* display reduced locomotory activity compared with healthy individuals. Reduced capacity for locomotion and increased mortality was observed by Zwölfer (1926a) in *G. pulex* infected by *T. mulleri*. Stempell (1902), however, did not find any apparent pathogenic effects due to these microsporidians. *S. mulleri* has no noticeable pathogenic effect on its hosts (Zwölfer, 1926a), as confirmed by my own observations. Two microsporidian species, *O. effeminans* and *T. herediteria*, associated with *G. duebeni* have no pathological effects. Infected and noninfected specimens of *G. duebeni* have been reared for many generations in the laboratory. Several life functions have been studied and compared, such as brood size, mortality of embryos, juveniles and adult individuals, growth intensity, onset of maturity, life span, molting frequency, and metabolic rates, with no significant differences observed between infected and healthy amphipods (Bulnheim and Vávra, 1968; Bulnheim, 1971a). Results of investigations on standard metabolism as a function of size in noninfected *G. duebeni* juveniles and females compared with those infected by *T. herediteria* are presented in Figure 4 (cf. Bulnheim, 1972b for further experimental details). Despite the rather severe infection of the host, only a slight reduction in the respiratory rate was established with increasing size.

Except for a reduced resistance to temperature stress noticed in specimens infected by *T. herediteria*, no further differences in the life functions tested have been revealed. Thus, the results obtained from studies on *O. effeminans* and *T. herediteria* document the relationships between parasite and host to be rather well balanced.

INFLUENCE ON SEX DETERMINATION

Another aspect of the host-parasite relationship to be considered is the influence on the determination and inheritance of sex. This effect, elucidated by several studies on microsporidian infections in the brackish-water crustacean *G. duebeni* (Bulnheim, 1967, 1969, 1970, 1971a, 1972a; Bulnheim and Vávra, 1968), represents a peculiarity without parallel among Microsporidia.

G. duebeni exhibits considerable deviations from the normal 1:1 sex ratio. Rearing experiments demonstrated females produced young of both sexes and other females which give birth only to female offspring. Females of such strains mated with males from strains of

different origin again produced broods consisting exclusively or predominantly of females, but accompanied by a few males or intersexes. Since this sex-ratio condition was transferred through the maternal line, cytoplasmatic inheritance was first assumed (Traut, 1962).

During an investigation conducted to find the suggested extra-chromosomal elements affecting sex determination, the occurrence of transovarially transferred microsporidians was detected. Since these were found almost exclusively in females, but sometimes in intersexes, it was supposed that the parasites might be involved in the mechanism of sex determination. In order to obtain definite proof of this assumption, the sex ratio of female progeny from a noninfected strain was compared with that from experimentally infected females. Females derived from one brood of two noninfected strains were divided into two groups. The females of one group were infected by inoculation of contaminated ovarial tissue into the body cavity, while the other group was not infected and served as a control. After pairing with males about 2 months later, offspring from the two groups were reared and sexed. The results of this experiment, listed in Table 2, clearly demonstrate that the experimental infection, compared with the control, resulted in shifting the sex ratio significantly toward females. The female descendants of the experimentally infected females proved to be contaminated and produced only unisexual offspring.

The microsporidian responsible for this sex-ratio condition was shown to be *O. effeminans* (Bulnheim, 1967; Bulnheim and Vávra, 1968). Later, another microsporidian, *T. herediteria*, was detected in *G. duebeni* females, which affects sex determination of its host in the same way when transmitted via the eggs to the offspring through successive generations (Bulnheim, 1969, 1971a). For this reason, males do not harbor these parasites.

It appeared of interest to obtain information on whether or not sex determination could be established in other amphipods infected with microsporidians. Therefore, the following amphipods were studied: *G. locusta*, *G. salinus*, and *G. pulex*. They were all infected with *Thelohania* sp. whose taxonomic position at the species level, as previously mentioned, is still uncertain. *Thelohania* sp. are transferred transovarially and, thus, the young are infected at birth before sexual differentiation is initiated. In *G. locusta* and *G. salinus*, breeding experiments with specimens attacked by the microsporidians were conducted in the laboratory, whereas parasitized individuals of *G. pulex* were collected in the field. In all cases studied, it became evident that females as well as males are associated with microsporidians. Therefore, a sex-determining influence by *Thelohania* sp. on their hosts can be excluded.

With regard to the particular host-parasite relationship detected in the two microsporidian species associated with *G. duebeni* females, the question arises: what is the underlying mechanism of the host's response to the infection? The suggestion that the microsporidians might be involved in influencing genetic processes controlling determination and inheritance of sex in *G. duebeni*, which was initially taken into consideration, could not be confirmed. Detailed studies on the mode of sex determination in this amphipod revealed a lack of heteromorphic sex chromosomes. From the results obtained by breeding experiments with non-infected individuals, it can be concluded that sex determination is governed by a balanced polygenic system of sex genes, whereby environmental factors such as different photoperiods may have a pronounced influence on sex ratio (Bulnheim, 1969, 1972a).

Considering the mode of the sex determining influence by *O. effeminans* and *T. herediteria*, the assumption is proposed that the microsporidians might affect the differentiation of the androgenic gland during postembryonic development. This gland, situated at the vas deferens, is the source of the male sex hormone in Malacostraca and controls the differentiation of primary and secondary male sex characters, and if it does not develop its hormonal function the gonads develop into ovaries (Charniaux-Cotton, 1956, 1965).

Observations on infected intersexes with rudimentary androgenic glands and, later on, from experimentally infected males, demonstrate that this organ or its anlage are not attacked by the parasites. Therefore, it is probable that any substances or byproducts the microsporidians excrete during their multiplication might inhibit the differentiation of this gland. The idea that the parasites might cause such inhibitory effects was supported by another finding. As mentioned above, males of *G. duebeni* have not been shown to be parasitized by either *O. effeminans* or *T. herediteria*. Therefore, experimental infection of adult males was undertaken in order to test whether it exerts an influence on their sexual organization. Since attempts at peroral transfer failed, an inoculation was accomplished by transplantation of contaminated tissues of females into the male body cavity. After several molts, the male test animals infected by *O. effeminans* exhibited some alterations in their external sex characters. It was found that the size of the gnathopods, uropods, and antennae was gradually reduced and development of oostegites, which represent secondary sex characters of females, was observed. Thus, the males attained an intersexual appearance with respect to external characters. However, the male gonads, although invaded by microsporidians, displayed no structural changes.

This finding indicates that interference of microsporidians with the male sex hormone produced by the androgenic gland might be responsible for the sexual alterations observed. On the other hand, a species-specific effect of the microsporidians upon their host

Table 2.—Results of breeding experiments (15°C, 10‰ salinity) in *Gammarus duebeni* infected with *Octosporea effeminans*.[1]

	Strain	No. of couples	No. of broods	No. of juveniles	Sex ratio ♀:♂	Mortality (%)
Uninfected controls	da	3	9	555	224:231	18
	db	7	25	1,066	215:665 94 intersexes	16
Infected amphipods	da	3	9	458	393:10	12
	db	7	25	650	568:0 3 intersexes	12

[1]Females divided from one brood of two noninfected strains (da, db) were divided into two groups. The females of the one group were experimentally infected by transplantation of contaminated ovarial tissue, whereas the other group served as a control to compare sex ratio of descendants in both series. (Data from Bulnheim, 1967).

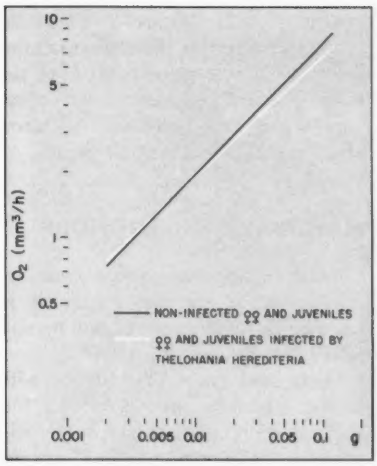

Figure 4.—Relationship between oxygen-uptake rates (standard metabolism) and size (wet weight) of *Gammarus duebeni* ♀♀ and juveniles infected by *Thelohania hereditaria* in comparison with ♀♀ and juveniles free of microsporidians (15°C, 10‰ S). Consumption of O_2 is expressed by the regression equations log y = log 24.35 + 0.57 log x (infected specimens) and log y = log 30.63 + 0.607 log x (noninfected specimens). There are no significant differences ($p>0.1$) between the slopes of the two regression lines.

cannot be excluded. This became obvious when *T. hereditaria*, experimentally transferred to *G. duebeni* males, did not induce any changes in either external or internal sex characters. The assumption of a species-specific action is confirmed by observations with *S. mulleri* in association with *G. duebeni*, where neither a sex-determining influence on the host nor a modification of sex characters occurs.

It should be mentioned that a hormonal involvement of a microsporidian has been detected in insects. In a study on endocrinological implications of *Tribolium* larvae infected by *Nosema* sp., Fisher and Sanborn (1964) demonstrated the ability of the parasites to produce a substance with juvenile hormone activity in their hosts.

The various aspects which pertain to the relationships between microsporidian infection and sex determination in *G. duebeni* cannot be discussed in full detail in this presentation. Additional results obtained from these investigations will be submitted in a forthcoming publication. In addition, the interrelations found between environmental factors, multiplication of the microsporidians, and sex differentiation of the host have not been outlined in this report, since recent studies provide detailed information on this subject (Bulnheim, 1969, In press).

Reference should be made to host-parasite interactions between some *Thelohania* sp. and mosquitoes. In *Culex tarsalis*, and probably also in other representatives of *Culicidae*, infection by *Thelohania* sp. is fatal to male larvae. Sporogony only occurs in male hosts which usually succumb with progressive infiltration by the parasites, whereas females acquire benign infections and harbor only schizogonic stages which are transmitted transovarially (Kellen and Wills, 1962; Kellen et al., 1965). The same responses have been reported in *Anopheles* sp. infected with *Thelohania legeri*, which is carried via the eggs to their progeny. In each generation the infected female larvae develop into adults, while infected male larvae die (Hazard and Weiser, 1968). In contrast to the situation found in the progeny of *G. duebeni* females associated with *O. effeminans* or *T. hereditaria*, the shift of sex ratio in mosquitoes is caused by selective mortality of the offspring during the larval stages.

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MFR PAPER 1147

Fine Structure of *Minchinia* sp. (Haplosporida) Sporulation in the Mud Crab, *Panopeus herbstii*

FRANK O. PERKINS

ABSTRACT—Morphogenetic changes which accompany sporulation of Minchinia *sp. in the mud crab (*Panopeus herbstii*) are described. Small (4-19 μm diameter) plasmodia, the precursor cells for the sporulation sequence, increase in size and numbers of nuclei per cell to form sporonts with a delimiting wall. Since widely divergent nuclear sizes (2.3-5.9 μm diameter) and paired small nuclei (<3 μm diameter) were observed in sporonts, it is suggested that karyogamy occurs followed by meiosis. Evidence for meiosis is the observation of synaptonemal-like complexes and polycomplex-like structures in sporont nuclei. Sporonts cleave into uninucleate sporoblasts following one of two pathways. In the presumably "normal" sequence, a cytoplasmic syncytium is formed followed by cleavage into uninucleate sporoblasts. In the other, sporont protoplasm is asynchronously "carved" into uni- or binucleate sporoblasts or sporoplasms as a result of delimitation at the surface and internally. Since aberrant spore formation was commonly observed in the latter type of sporont, its cleavage patterns are considered to be anomalous. A sporoplasm is then delimited in each sporoblast and spore maturation follows. Haplosporosomes were observed in plasmodia and spores, but not in intermediate cell stages. Mitotic apparati appear to be persistent through interphase in plasmodia and sporonts and consist of two spindle pole bodies connected by a bundle of microtubules.*

INTRODUCTION

A species of *Minchinia*, parasitizing the mud crab, *Panopeus herbstii*, was obtained from the York River, Virginia estuary. Collection sites were intertidal and subtidal in the salinity range of about 6 to 22‰. The haplosporidan closely resembles *Minchinia louisiana* (Sprague, 1963) except that spores of the Louisiana species average 12.1 μm long by 8.4 μm wide whereas those of the Virginia species average 9.6 × 8.1 μm (N = 30; range = 9.0-10.0 × 7.5-9.0 μm). Therefore, since spore size is considered to be an important species characteristic used in haplosporidan taxonomy (see for examples: Couch, 1967; Sprague, 1963; Wood and Andrews, 1962), the present sporozoan will be considered as *Minchinia* sp.

The ultrastructure of spore formation in other closely related Haplosporida has been studied by Perkins (1968; 1969; 1971) and Ormières et al. (1973). Spore fine structure of several species has been described by Ormières and de Puytorac (1968) and Rosenfield et al. (1969). One of the species considered by the latter authors was *Minchinia* sp. found in the mud crabs, *Eurypanopeus depressus* and *Rhithropanopeus harrisi*, about 100 miles north of the York River. They also noted its similarity to *M. louisiana*, but reserved judgement on a species designation until further study. No spore dimensions were given.

This paper is concerned with the fine structure of sporulation in the third or possibly fourth species of *Minchinia* thus far studied at the ultrastructural level. Special attention is given to the extraspore wall ornaments, haplosporosomes, and implications of the ultrastructural data in life cycle studies.

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MATERIALS AND METHODS

The fine structure of *Minchinia* sp. sporulating stages was examined in samples of hepatopancreas and musculature from five specimens of *Panopeus herbstii* mud crabs. Two crabs with heavy infections of plasmodia, but without sporulation stages, were also examined. One mm^3 blocks of minced organs were fixed 30 min in 2.5 percent glutaraldehyde buffered at pH 7.2-7.4 with 0.2 M Millonig's phosphate buffer. After three 10 min rinses in the same buffer with 0.15 M NaCl, the blocks were postfixed for 3 h in 1 percent OsO_4 containing 0.1 M Millonig's phosphate buffer (pH 7.4) and 0.2 M NaCl. All solutions were 22-24°C. Dehydration was accomplished in a graded series of ethyl alcohol and embeddings were made in Epon 812[1]. Sections were stained 45 min in a saturated aqueous solution of uranyl acetate followed by 10 min in Reynolds lead citrate. Permanganate fixations were made in 1.2 percent $KMnO_4$ in estuarine water (ca. 20‰ salinity) for 10 min followed by dehydration and embedding as described above.

Whole mounts of spores were prepared for scanning electron microscopy by shaking minced hepatopancreas in estuarine water, centrifuging to separate spores from other cells and cell particulates, fixing in the glutaraldehyde solution described above, and rinsing in distilled water. Rinsed spores were placed on a coverslip fragment, immersed in liquid Freon 22 for 15 sec, then into liquid nitrogen followed by freeze drying on a brass block which had been temperature equilibrated in liquid nitrogen. The spores were then shadowed with gold-palladium and examined at 25 KV.

For light microscopy infected crab organs were placed in Davidson's fixative (Shaw and Battle, 1957) for 2-3

[1]Mention of trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

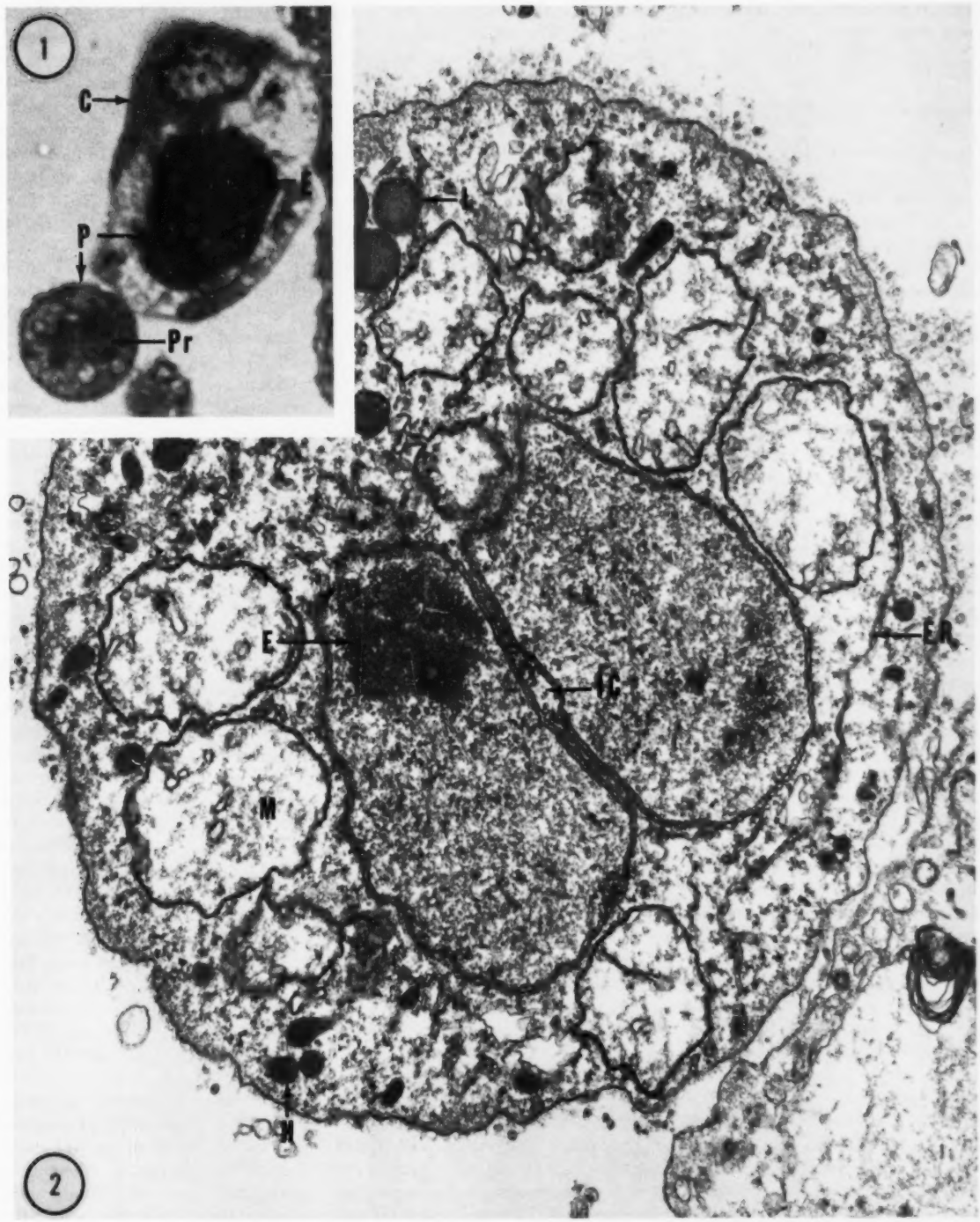


Figure 1[2].—Brightfield micrograph of *Minchinia* sp. plasmodia (P). Crab haemocyte (C); endosome (E); paired nuclei (Pr). 2,200×. Figure 2.—Plasmodium with paired nuclei. Endosome (E); haplosporosome (H); mitochondrion (M); lipoid droplet (L); internuclear chamber (IC); smooth endoplasmic reticulum (ER). 28,000×.

days and embedded in Paraplast (Sherwood Medical Industries, Inc.). Sections were stained in Harris' haematoxylin and counterstained in eosin Y. Measurements of sporont nuclei were made to the nearest 0.2 μm using 3 μm thick sections of the Epon-embedded material stained in aqueous 1 percent toluidine blue adjusted to pH 11 with 1 N NaOH. Plasmodial nuclei were measured to the nearest 0.2 μm using sections of paraffin-embedded material.

[2]Unless otherwise indicated all figures are electron micrographs and all specimens were fixed in glutaraldehyde followed by osmium tetroxide.

Spores were measured from living cells in fresh preparations.

RESULTS

Plasmodia

Presporulation cell types consist predominately of approximately spherical or spheroidal, uni- or binucleate plasmodia without a delimiting wall and measuring from 4.0 to 9.0 μm in longest axis after fixation. The largest plasmodia contained up to 24 nuclei and were as large as 19 μm in longest axis after fixation. Nuclei of all plasmodia were from 1.6 to 8.0 μm diameter (N = 94). Only 14 percent of the nuclei were greater than 3.0 μm diameter (Fig. 10). Those which were about 4.0 μm or larger had nucleoplasm of low staining density in haematoxylin- and eosin-stained sections. Whether they represented nuclei which enlarged during fixation in Davidson's or were reflections of the actual sizes was not determined. They were never observed in glutaraldehyde- and osmium tetroxide-fixed cells; however, that may be due to the smaller numbers of cells seen in the Epon-embedded material. A few of both the 2.2-3.0 μm size nuclei and the large vesicular-type nuclei were observed to be in division.

Generally the nuclei of binucleate plasmodia were found pressed together (Figs. 1-3) with the nuclear envelopes separated by a 30-36 nm wide zone along the opposed surfaces except near the center where the envelopes are infolded forming a cytoplasmic chamber as wide as 1.1 μm between envelopes (Fig. 3). Between opposed portions of the envelope, cisternae of the smooth endoplasmic reticulum were often found. They were generally small vesicles or fragments of flattened cisternae. Although it was not illustrated in previous papers (Perkins, 1968; 1969; 1971), I observed the same structural characteristics for paired nuclei of *M. nelsoni*, *M. costalis*, and *U. crescens*. A single, generally spherical, and Feulgen-negative endosome was found against the nuclear envelope of *Minchinia* sp. Secondary clumps of basophilic, Feulgen-positive material were scattered in the nucleoplasm.

The cytoplasm included lipid-like droplets, mitochondria, and a sparse array of smooth endoplasmic reticulum (Fig. 2). An anastomosing reticulum of cisternae was also found in many cells (Fig. 3). Whether it represented a modified Golgi body was not determined. Haplosporosomes (Figs. 2-5) (Perkins, 1971) were scattered throughout the cytoplasm as already observed in *M. nelsoni*, *M. costalis*, and *U. crescens*. The inclusions of *Minchinia* sp. did not appear to arise from generative regions as was presumed to occur in *M. nelsoni* (Perkins, 1968) nor were the haplosporosomes similar in shape to those in other haplosporidans. Those of *Minchinia* sp. were club-shaped (Fig. 4) with the head of the club ranging from 129 to 186 nm diameter ($\bar{X}$ = 155; N = 11; $S_{\bar{X}}$ = 6); the "handle" diameter, from 84 to 117 nm ($\bar{X}$ = 95; N = 7; $S_{\bar{X}}$ = 5); and the overall length, from 300 to 586 nm ($\bar{X}$ = 456; N = 7; $S_{\bar{X}}$ = 37). It is not known, however, whether some of the profiles which were assumed to be cross-sectional views could have represented medial sections of spheres or spheroids. The haplosporosomes had a three-part substructure consisting of two electron-dense zones separated by a thin electron-light zone as already described in the other species and were delimited by a unit membrane. The internal electron-light zone proved to have a tripartite substructure when fixed in potassium permanganate (Fig. 5). Each plasmodium had fibrogranular material adhering to the surface (Fig. 6), but no wall as appears during sporulation (Fig. 8).

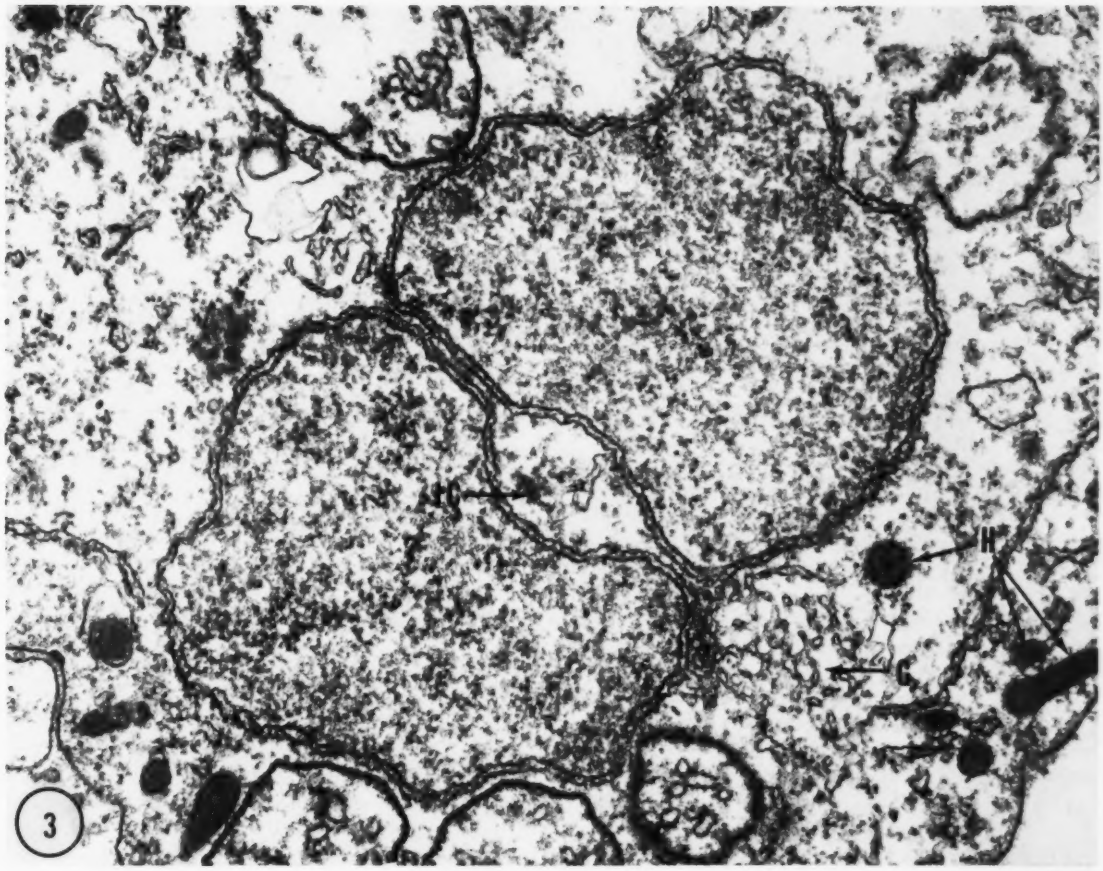
Nuclear division occurs without nuclear envelope breakdown and with the involvement of a persistent mitotic apparatus. The latter was found in 76 percent of 75 nuclear profiles counted; therefore, it is assumed that interphase nuclei contain the apparatus. A positive sighting was considered to be a group of five or more microtubules. Only nuclei that did not show evidence of lengthening and pinching in half were counted. The mitotic apparatus consists of two spindle pole bodies embedded in the nucleoplasm free of the nuclear envelope or in some cases touching on the envelope, but not embedded in the envelope or in the cytoplasm. A bundle of microtubules connects the two bodies (Fig. 7). Upon division the nucleus changes from a sphere to a spheroid, lengthens further into a dumbbell-shaped unit, then pinches in half. The microtubular bundle lengthens as division proceeds.

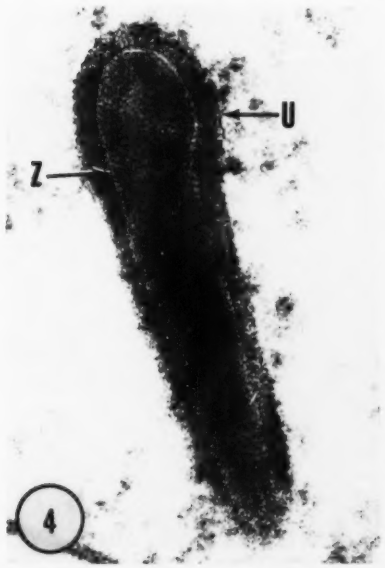
Sporont

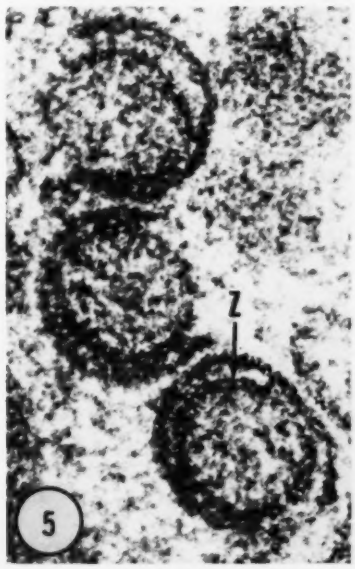
Upon initiation of sporulation, the plasmodia increase in mass and number of nuclei. Whether this is a result of plasmogamy was not determined. A wall about 23 nm thick is formed around each cell and persists until spore maturation and release (Fig. 8). In spite of its size, the wall can be seen in the light microscope when the cytoplasm separates from it, because adhering fibrogranular material increases the apparent thickness and both "layers" stain deeply in toluidine blue (Figs. 16 and 17). Upon addition of the wall, the plasmodia are herein considered to have developed into sporonts until sporoplasm delimitation occurs at which time they are termed sporocysts. Sporonts were 22-71 μm (N = 20) in longest axis and had the same general shape as plasmodia. The cytoplasm resembled that of plasmodia except that haplosporosomes disappeared from the cytoplasm and few lipoid bodies were observed.

Sporont nuclear division was identical to that in plasmodia and the division apparatus (microtubules and spindle pole bodies) appeared to persist through interphase as in plasmodia. Although nuclear division figures were observed, it was not determined how many division cycles occur after the cells become sporonts. It is suspected, however, that at some point karyogamy occurs followed by meiosis. Nuclear sizes varied from 2.3 to 5.9 μm diameter (N = 275) with most individual sporonts containing nuclei of one limited size range ($\pm$ ca. 0.5 μm) not mixed sizes. However, a few sporonts had large nuclei and small paired nuclei in the same cell. In Figure 9 there is one pair of small nuclei (2.5 and 2.9 μm) and five larger nuclei (4.1-4.6 μm; $\bar{X}$ = 4.4 μm). The section shown in Figure 9 was one of a series, thus the possibility of measuring significantly less than the full nuclear diameter was eliminated. When nuclear pairing was observed it nearly always involved small (< 3.0 μm) nuclei. "Pairing" is considered to exist when the opposed surfaces of paired nuclei are flattened as in Figures 1 (lower pair of nuclei) and 2.

Since there appeared to be a possibility that karyogamy occurs in the sporonts, nuclear diameters were examined in an effort to see if a bi- or trimodal size







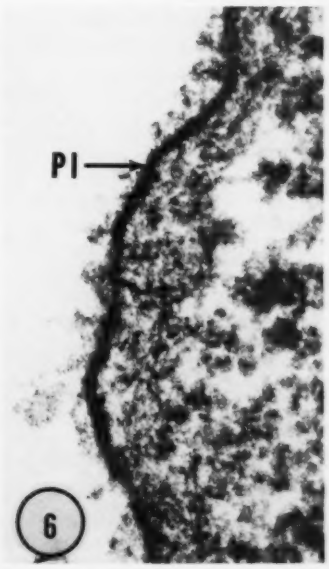
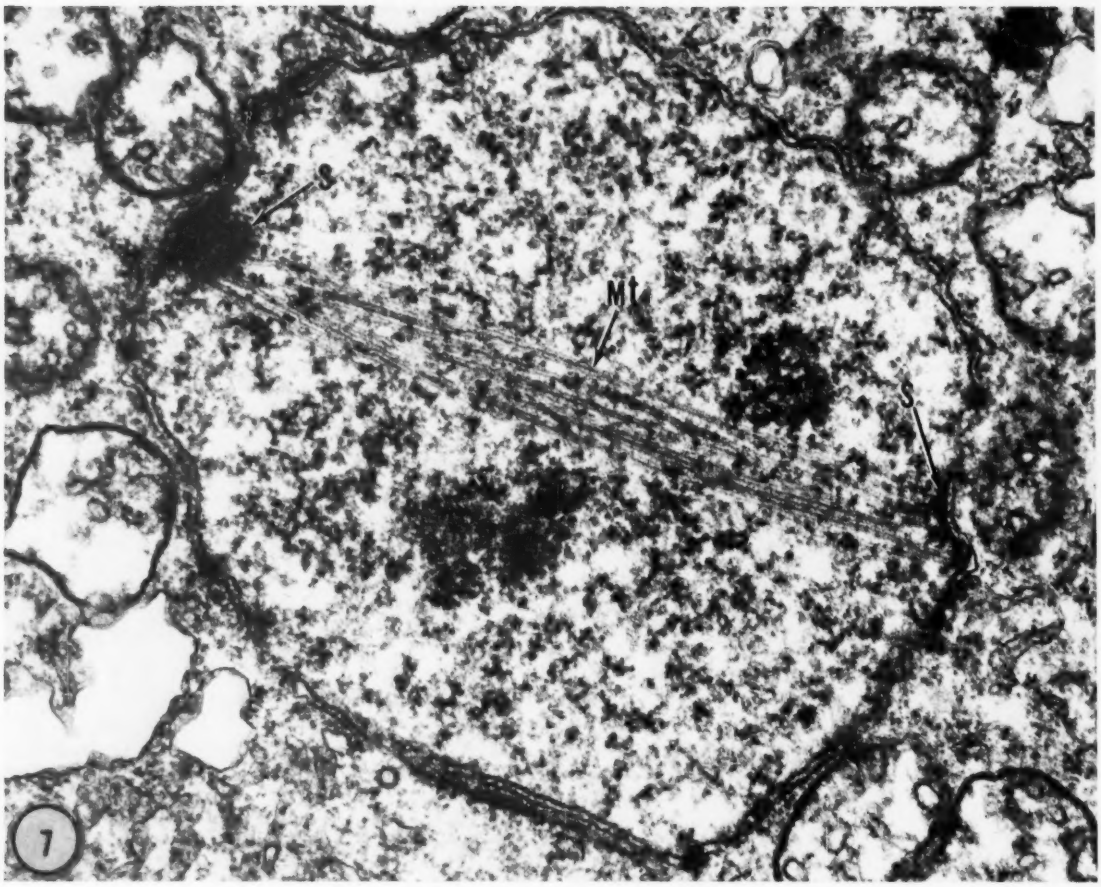
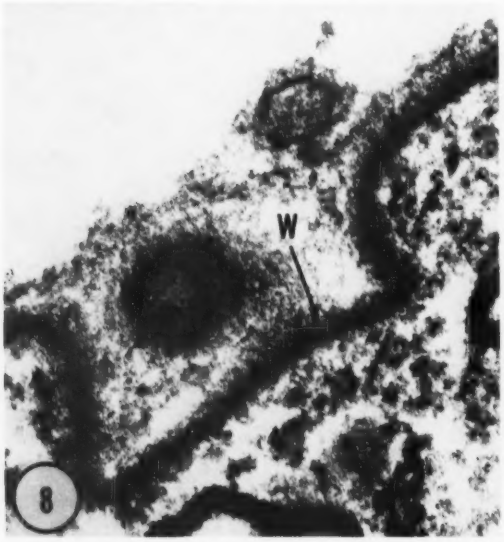


Figure 3.—Paired nuclei in plasmodium. Note internuclear chamber (IC); haplosporosomes (H); Golgi-like body (G). 35,000×. Figure 4.—Haplosporosome in plasmodium. Note characteristic substructure consisting of two electron-dense regions separated by an electron-light zone (Z). The inclusions are delimited by an unit membrane (U). 168,000×. Figure 5.—Haplosporosomes fixed in potassium permanganate. Note that electron-light zone (Z), seen in Figure 4, appears to have a tripartite substructure like a unit membrane. 180,000×. Figure 6.—Plasmalemma (PI) of plasmodium. 127,000×.





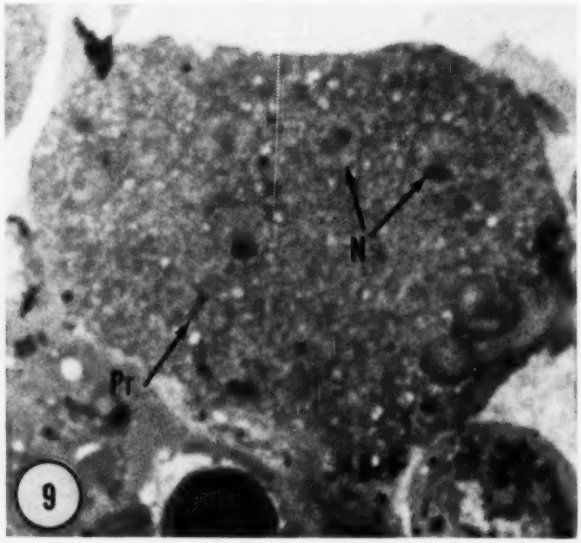


Figure 7.—Mitotic apparatus of plasmodial nucleus consisting of two spindle pole bodies (S) connected by a bundle of microtubules (Mt). Spindle pole body on the right is tangentially sectioned. 36,000×. Figure 8.—Plasmalemma and wall (W) of uncleaved sporont. 110,000×. Figure 9.—Brightfield micrograph of sporont in which a pair of small nuclei (Pr) and several large nuclei (N) are visible. It is believed that at this stage pairing of small (2.5-3.0 μm) nuclei occurs followed by karyogamy to yield larger (4-4.5 μm) nuclei. 2,100×.

distribution could be observed; however, when 275 nuclear diameters from 50 sporonts were represented in histograms, no clear evidence of polymodal distributions was observed, only a skewed distribution with one obvious mode. One such histogram with the data plotted at 0.2 μm intervals is shown in Figure 10. Two modes may be present, but they are not definitive.

Structures presumed to be synaptonemal complexes (SC's) and polycomplexes (PC's) were observed in sporont nuclei (Figs. 11-13). Thus meiosis is suspected to occur. The presumptive medial complex or ribbon appeared as a granular band in some sections (Fig. 11) (group B type of SC; Wettstein and Sotelo, 1971), or as a multilayered structure (Fig. 12) (group C type of SC). Differences in structure may have been due to variations in resolution and fixation quality. The medial space between lateral elements was about 67 nm wide. SC-like structures were not observed in nuclei of plasmodia, sporocysts, or spores, nor were they seen in paired or obvious anaphase and telophase nuclei of sporonts (Fig. 14).

Polycomplex-like structures consisted of from 3 to 9 electron-dense bands in parallel arrays separated by electron-light zones 15-51 nm wide (N = 9; $\bar{X}$ = 24; $S_{\bar{X}}$ = 4). The spaces varied greatly in size from complex-to-complex but within a given complex the spacing varied little (Fig. 13). The dense bands were 44-50 nm thick (N = 9; $\bar{X}$ = 46; $S_{\bar{X}}$ = 0.8). Complexes were found only in sporont nuclei and never in nuclei with the presumptive SC's. Due to the problems associated with identifying nuclear size from thin sections, it is not known whether the suspected SC's and PC's were found only in large (4 to 6 μm diameter) nuclei; however, it is known that at least some of the nuclei were in that size range (Fig. 15).

After sporont cleavage is initiated no further nuclear divisions occurred. Cytokinesis occurred in two ways, both of which were commonly observed. The more ordered pattern of cleavage resulted from peripheral and internal partitioning to yield a syncytium (Fig. 16). Further cleavage resulted in approximately spherical, uninucleate sporoblasts. Another type of cleavage which appeared to be aberrant, but which was observed as frequently, consisted of cleavage and delimitation of approximately spherical, uni- or, sometimes, binucleate sporoblasts and sporoplasms from the sporont at large. Delimitation occurred at the sporont periphery as well as internally thus forming free cells within the sporont wall, cells in invaginations of the sporont surface, and cells in vacuoles within the sporont (Figs. 17-20). Since synchronous delimitation does not occur, numerous stages of development are observed within a given sporont—from nearly mature spores to cleaving multinucleated fragments of sporont cytoplasm (Fig. 20). Obviously aberrant as well as a few normal-appearing spores (Figs. 17 and 20) were formed in these sporonts; therefore, the first cleavage sequence described above appears to result in formation of most of the spores since the sporocysts with large numbers of mature spores rarely contained any aberrant ones.

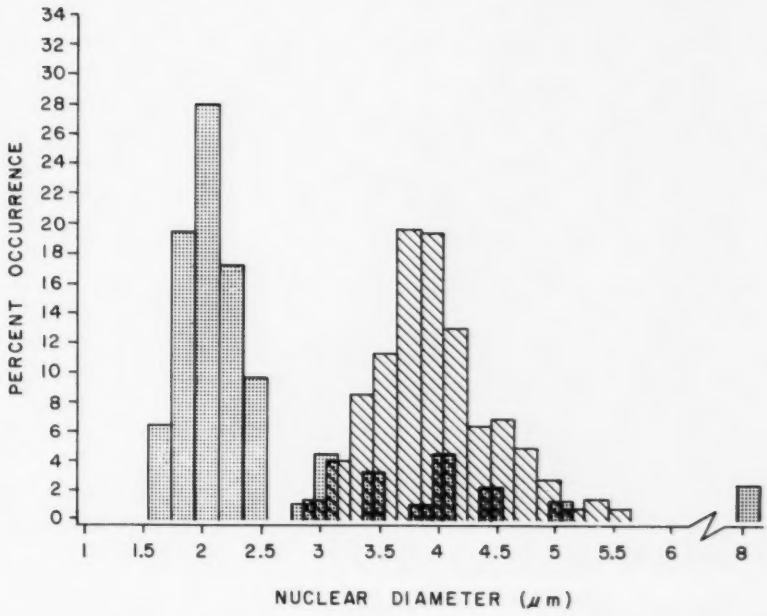
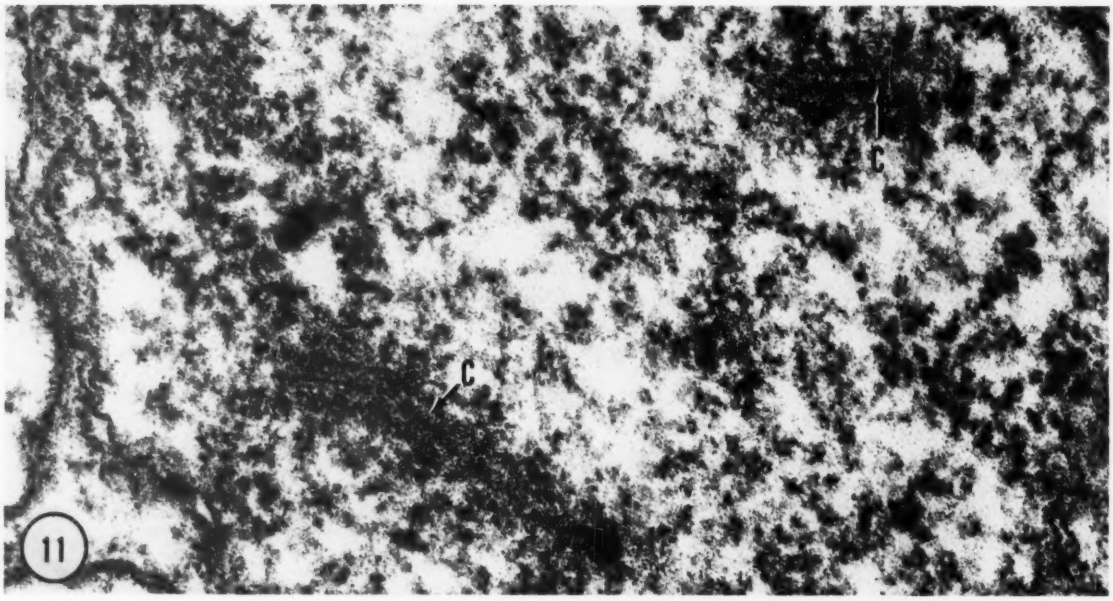


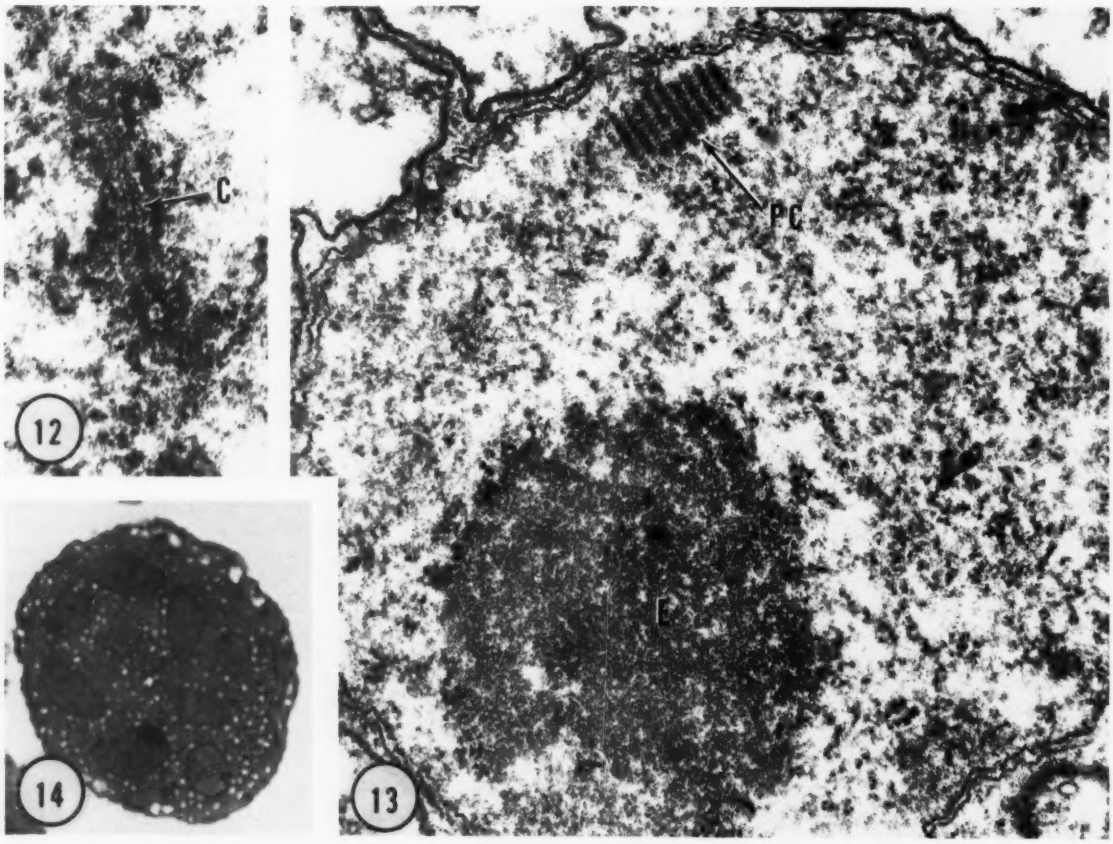
Figure 10.—Histogram showing distributions of nuclear diameters of plasmodia (dotted bars) and sporonts (hatched bars).

Sporocyst

Upon delimitation of the sporoplasm within sporoblasts the sporont is herein considered to be a sporocyst. I was not able to determine how separation of sporoplasm from the rest of the sporoblast cytoplasm occurred. Presumably it was formed by invagination of the sporoblast plasmalemma and/or fusion of cytoplasmic vesicles. Partial separation of sporoblasts into sporoplasm primordia and "envelope" primordia was not observed nor were degenerate nuclei seen; therefore, the scheme proposed by Ormières et al., (1973) is probably not descriptive of sporoplasm delimitation in *Minchinia* sp. (see "Discussion"). Cup-shaped units of anucleate cytoplasm, each partially surrounding a sporoplasm primordium, were observed in *Minchinia* sp., but their subsequent development was not determined.

As noted in my previous papers, spore wall formation occurs in the extrasporoplasm cytoplasm. A thin layer of electron-dense material was deposited on the outer surface of the extrasporoplasm cytoplasmic membrane which parallels the sporoplasm plasmalemma (Fig. 21). Subsequent additions of material yielded a laminated thick wall, then a final layer of electron-dense material was added to yield a corrugated surface (Fig. 22). The outermost layer appeared to form by fusion of long, 44-67 nm diameter ($\bar{X}$ = 55; N = 20; $S_{\bar{X}}$ = 1.4) strands of electron-dense material to the laminated spore wall or possibly from material similar to the strands, deposited in some other form (Fig. 23). The strands were apparently formed in vacuoles in





Figures 11 and 12.—Synaptonemal complex-like structures in sporont nuclei. Medial complexes (C) in Figure 11 are granular bands and the one in Figure 12 is multilayered. Figure 11: 49,000×; Figure 12: 70,000×. Figure 13.—Polycomplex-like structure (PC) in sporont nucleus. Endosome (E). 35,000×. Figure 14.—Brightfield micrograph of sporont showing nearly synchronous division of large (4-5.5 μm) diameter nuclei. 1,200×.

the extrasporoplasm cytoplasm. Vacuolar contents first appeared as a fibrogranular mass which then differentiated into parallel arrays of ca. 22 nm diameter tubules (Fig. 24). It is suspected that the latter then developed into the strands or wrappings, but intermediate stages were not visualized. Strands were either fused to the spore wall or wrapped loosely around the wall. Surface views of the mature wall (Fig. 25) showed strands as units of considerable length (greater than 10 μm) when unwound. Distally the strands appear to be about 50 percent of the proximal diameter, but this was not detected in the sectioned material.

Anteriorly the spore wall was flared forming a flange which was continuous at one side with a lid, as previously described in the other species of *Minchinia*. The lid rested on the flange and covered a pore in the spore case (Fig. 26). Thus the lid was hinged along one side (Fig. 26) and not continuous with the flange along the opposed side (Fig. 27). The plasmalemma delimiting the extrasporoplasm cytoplasm was invaginated at the non-hinge side (Fig. 27) and was continuous with the membrane opposed to the whole spore wall. The plasmalemma of the sporoplasm was not continuous with the membrane which was opposed to the wall. Interconnecting strands connected the flange and lid at the non-hinge side until later development led to partial fusion of lid and flange (Fig. 22).

By the time of sporoplasm delimitation, the Golgi body had appeared at the nascent anterior end of the spore. As previously described in other species (Perkins, 1968, 1969, 1971; Rosenfield et al., 1969), it consisted of convoluted, anastomosing cisternae which initially contained little or no dense material. As spore maturation proceeded, the cisternae filled with electron dense material in some regions. The material was released into the cytoplasm as membrane-bound inclusions with parallel bands of electron-dense material (Fig. 28). Haplosporosomes reappeared in the cytoplasm of developing spores from the membrane-bound inclusions, apparently budding from the periphery of the inclusion, thereby deriving a delimiting membrane from the inclusion membrane (Figs. 28 and 29). Haplosporosomes were pyriform, not club-shaped as in the plasmodia, and were

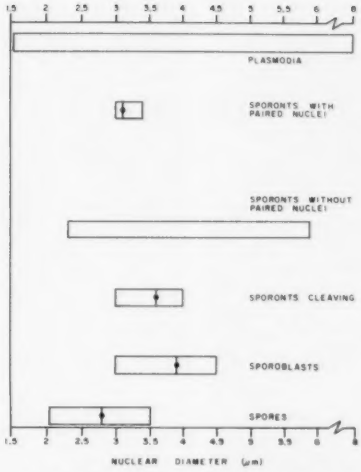


Figure 15.—Comparison of ranges and means of nuclear diameters of various cell types in sporulation sequence. No mean is given for uncleaved sporonts, because it is suggested that karyogamy followed by meiosis occurs in the sporonts; thus the nuclei of several cell stages have been unavoidably measured as one stage. The mean for plasmodia was omitted, because of uncertainties concerning the significance of the large vesicular nuclei (see "Discussion" section).

104-151 nm diameter ($\bar{X}$ = 124; N = 20; $S_{\bar{X}}$ = 2.9) through the globose portion. Both formative inclusions and free haplosporosomes were concentrated in the equatorial region of the spore. A few lipoid bodies, mitochondria, and cisternae of smooth endoplasmic reticulum were scattered throughout the cytoplasm.

There was only one nucleus per sporoblast, developing spore, and mature spore except in rare, presumably aberrant, cases where two were observed in the first two stages, but not in mature spores. The two nuclei were often in a paired configuration as seen in developing sporonts; therefore, the possibility that pairing does not represent prekaryogamy orientation must be considered. However, binucleate sporoblasts and spores were seen only in sporonts cleaved in the presumably aberrant manner (see previous "Sporont" section). Nuclear size decreased back to approximately that of the most commonly observed, non-vesicular plasmodial nuclei (Fig. 15). The mitotic apparatus, persistent in plasmodial nuclei up to sporoblast delimitation, was not in spore nuclei or in sporoblasts where sporoplasm delimitation had occurred.

As with the other species of *Minchinia* and *Urosporidium* studied, spore maturity yielded spores with high electron density which were relatively impervious to fixatives and embedding media; therefore, fully mature sporoplasms were not viewed in detail in sectioned material. Freeze-etch replicas indicated that little further structural modification in the sporoplasm occurred, only dehydration as evidenced by degree of sublimation during freeze-etching.

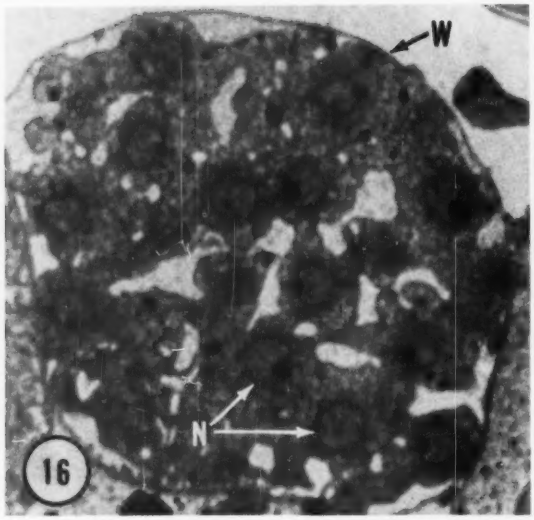
In interference optics the nucleus, Golgi body, and concentration of inclusions were visible in living spores (Fig. 30). The three were also visible in sectioned, stained spores (Fig. 31).

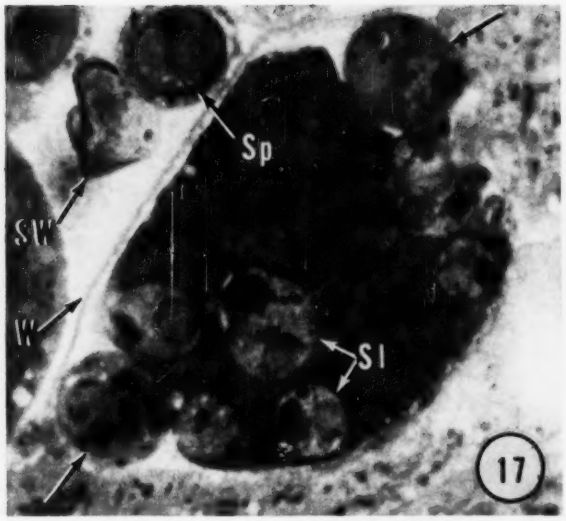
After formation of uninucleate sporoblasts, the sporoplasm is delimited followed by spore wall formation then loss of the extrasporoplasm cytoplasm except for the wall wrappings (Fig. 25). The fate of the spores is unknown.

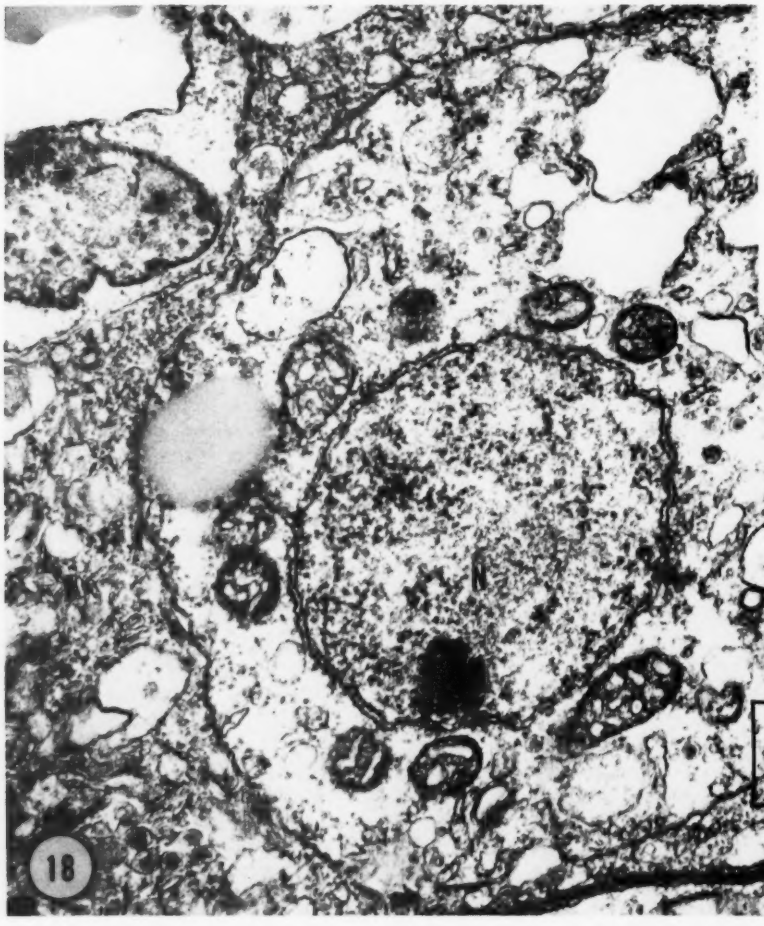
DISCUSSION

As Andrews (1966) has noted in his extensive epizootiological studies of *Minchinia nelsoni*, the life cycle of this oyster pathogen is poorly known. An important factor in this dearth of information is inability thus far to identify the reservoir of infective elements. The present study was, in part, initiated to determine whether the fine structure of *Minchinia* species in *Panopeus herbstii* resembled *M. nelsoni*. The mud crab is commonly found associated with oysters and, therefore, is a logical organism to consider as an alternate host.

Evidence from the comparative fine structure of the two parasites indicates that they are distinct species. They differ in the following respects: 1) *M. nelsoni* plasmodia have large formative regions for haplosporosomes (Perkins, 1968), none are seen in *Minchina* sp.; 2) haplosporosomes are club-shaped in *Minchinia* sp., spherical in the oyster pathogen; 3) large numbers of haplosporosomes are found in spores of the mud crab parasite, but none were readily identified in spores of *M. nelsoni* although electron-dense bodies were present which showed a faint indication of the usual bipartite substructure typical of haplosporosomes; and 4) the strands or wrappings around the spores of *M. nelsoni* were tubular (43-63 nm; $\bar{X}$ = 50 nm diameter) with a cartwheel-like internal substructure, whereas those of *Minchinia* sp. were electron-dense strands with no discernible substructure although the diameters were similar. At







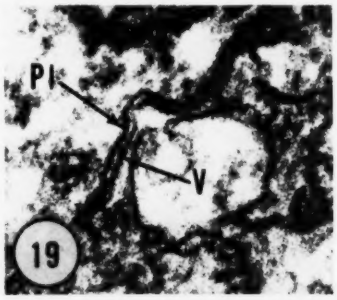
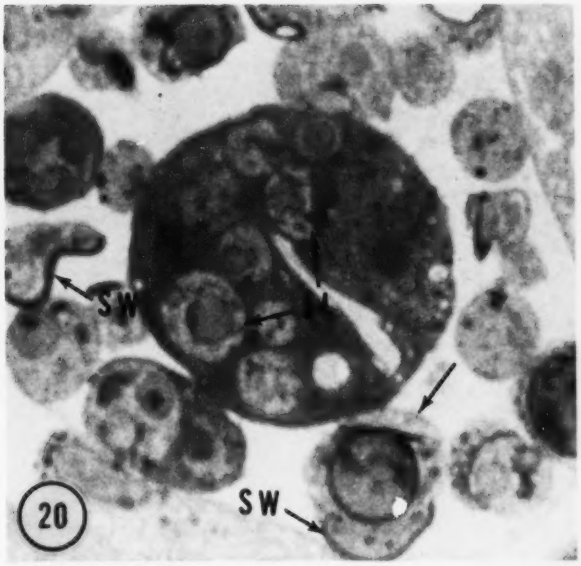
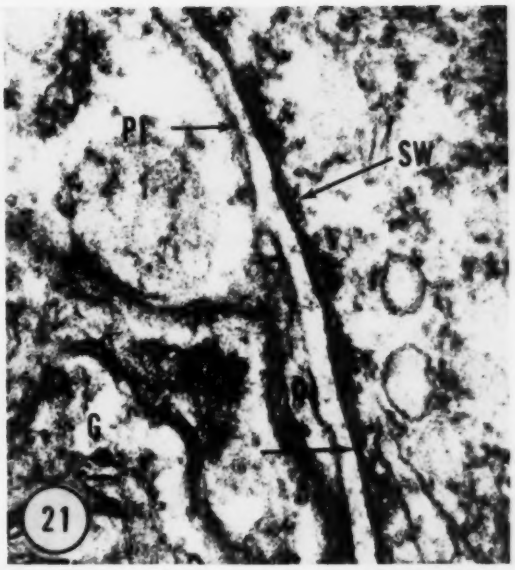
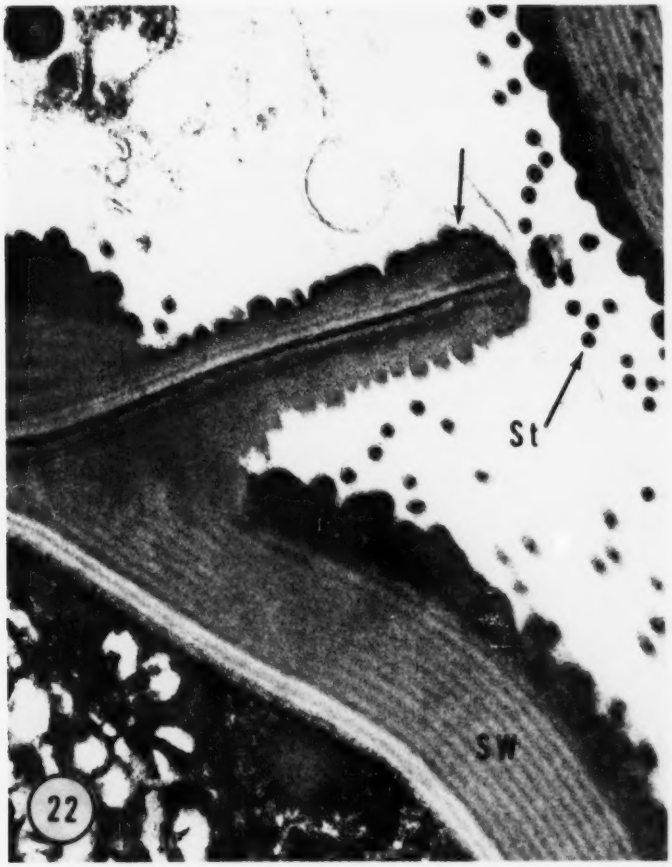


Figure 16.—Multinucleate syncytium resulting from partial internal and peripheral cleavage of sporont. Nuclei (N); sporont wall (W). 1,700×. Figure 17.—Sporont showing aberrant (?) cleavage which yields free uninucleate cells (arrows) and fully delimited sporoplasms (SI). Sporont wall (W); developing spore (Sp); aberrant spore wall (SW). 1,600×. Figs. 18 and 19.—Sporoplasm delimited within sporont which showed aberrant (?) cleavage patterns. Subdivision into sporoblasts had not occurred. Figure 19 is higher magnification of area within square showing sporoplasm plasmalemma (PI) and membrane (V) of vacuole in which sporoplasm is situated. Sporoplasm nucleus (N). Figure 18: 29,000×; Figure 19: 90,000×.







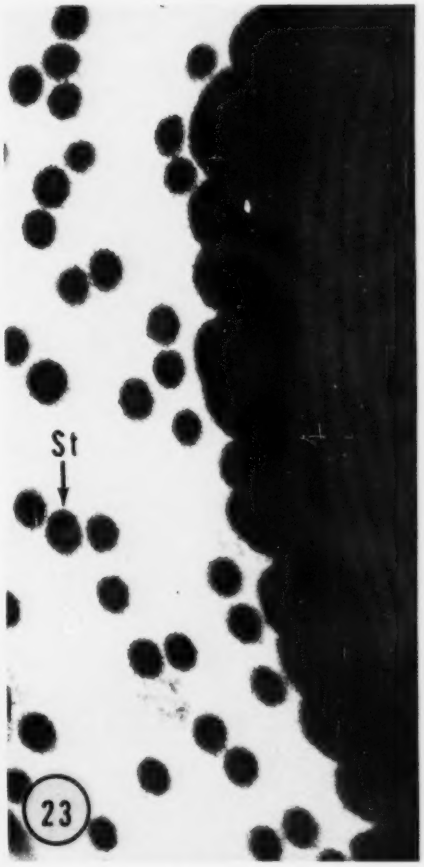
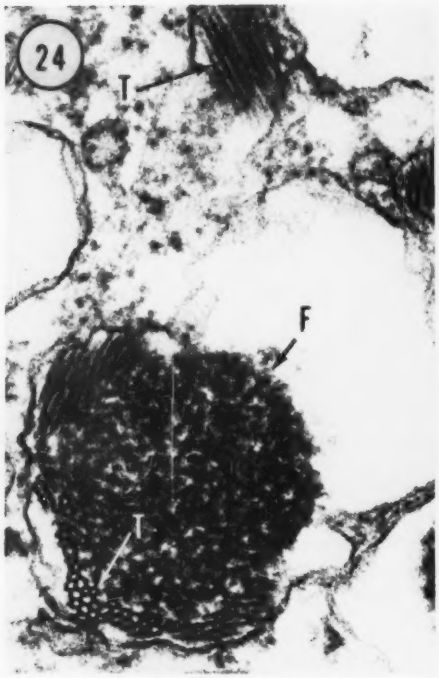
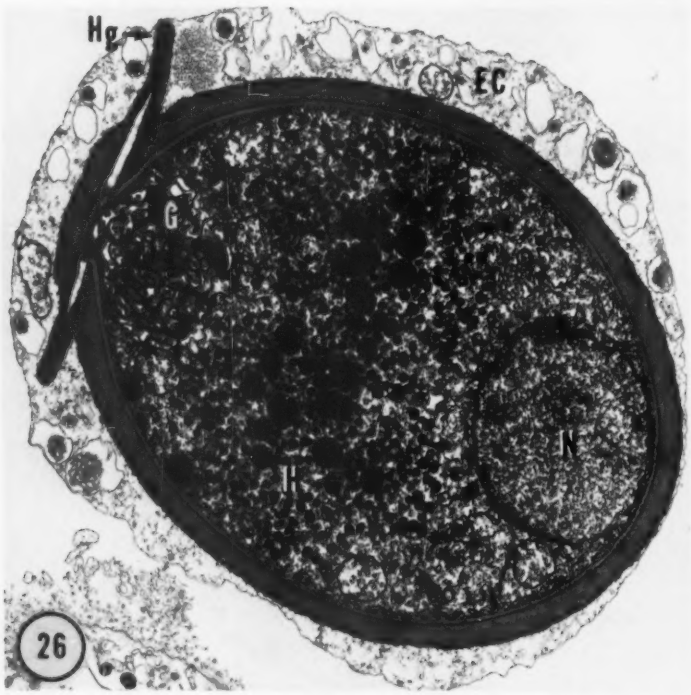


Figure 20.—Brightfield micrograph of cleaving aberrant sporont. Sporoplasms (Sl) have been cleaved from largest mass of cytoplasm. Nearly mature spore (arrow); aberrant spore wall formation (SW). 1,800×. Figure 21.—Initiation of spore wa.. (SW) formation. Plasmalemma of sporoplasm (Pl); membrane of extrasporoplasm cytoplasm (arrow); Golgi body (G). 77,000×. Figure 22.—Mature spore wall (SW) showing unhinged portion of lid-flange complex (arrow). Strands or wrappings around spore wall (St). 47,000×. Figure 23.—High magnification of spore wall showing corrugated surface and cross sections of strands (St). 106,000×.





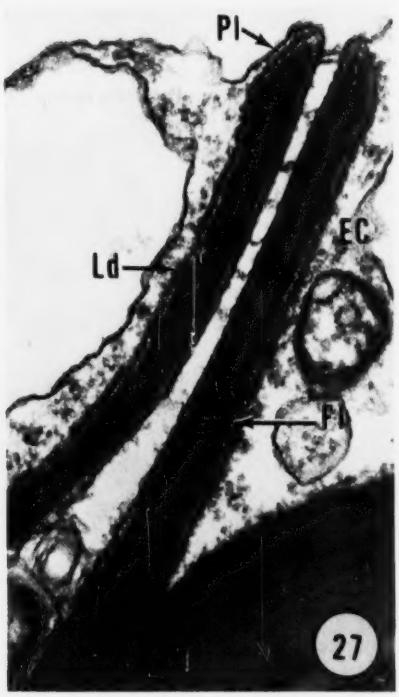
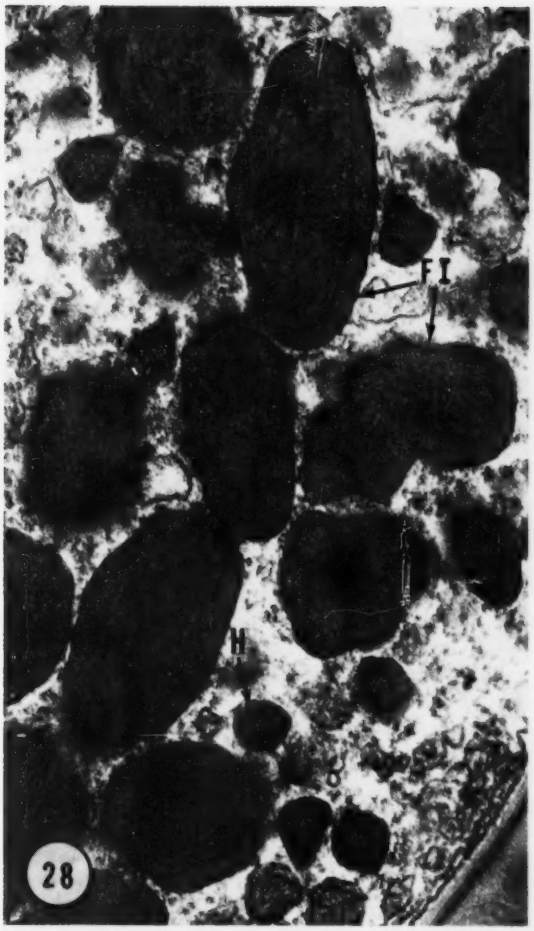
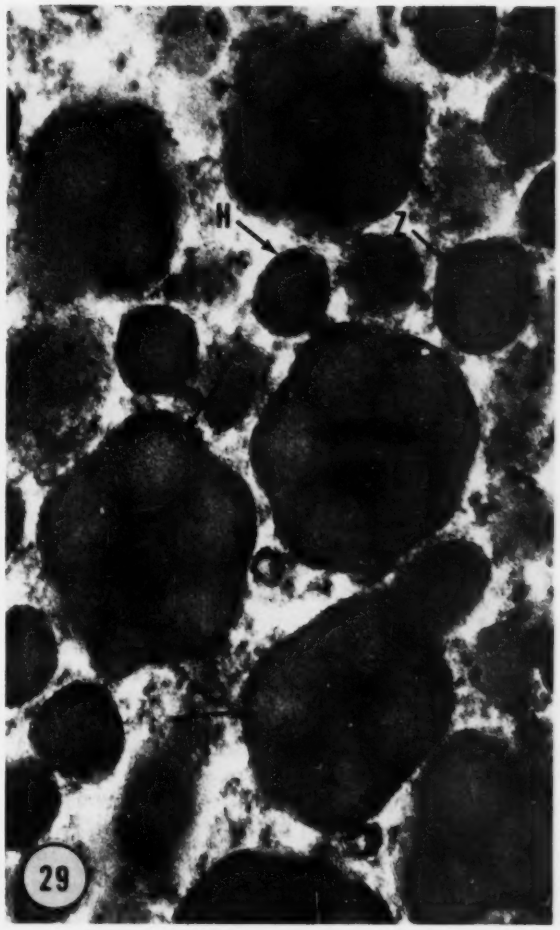
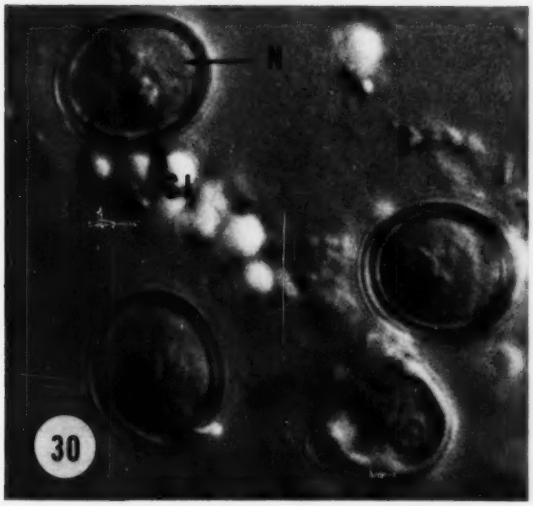


Figure 24.—Fibrogranular material (F) in vacuoles of extrasporoplasm cytoplasm. It is suggested that the material is used in synthesis of the tubules (T) which, in turn, are used in formation of the strands seen in Figures 22 and 23. 70,000×. Figure 25.—Scanning electron micrograph of spore. Note strands (St) or wrappings around spore wall. 5,800×. Figure 26.—Immature spore sectioned through hinge (Hg) region of lid-flange complex. Nucleus (N); haplosporosomes (H); Golgi body (G); extrasporoplasm cytoplasm (EC). 13,000×. Figure 27.—Non-hinge region of lid-flange complex. Note that plasmalemma (Pl) of extrasporoplasm cytoplasm (EC) invaginates and lies at the interface between lid (Ld) and flange (Fl). 57,000×.







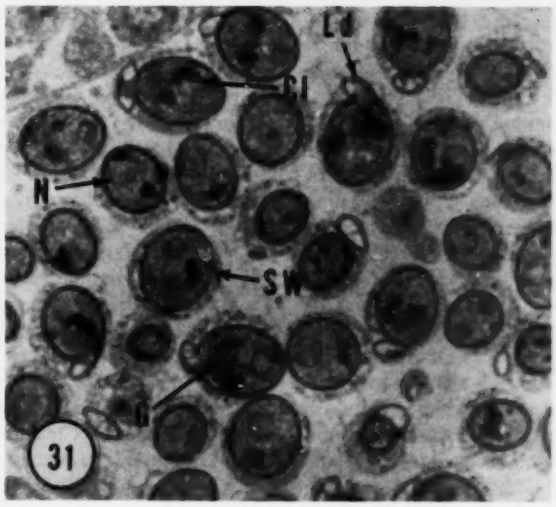


Figure 28.—Formative inclusions (FI) of immature spore in which haplosporosomes (H) are assembled. Inclusions are formed in Golgi body. 66,000×. Figure 29.—Higher magnification of formative inclusions showing haplosporosomes (arrows) being formed. Free, fully-formed haplosporosome (H); electron-light zone (Z) (see Figures 4 and 5). 104,000×. Figure 30.—Nomarski interference micrograph of living spores. Golgi body (G); nucleus (N); cluster of haplosporosomes and formative inclusions (CI). 1,000×. Figure 31.—Brightfield micrograph of sporocyst showing developing spores. Spore wall (SW); nucleus (N); cluster of haplosporosomes and formative inclusions (CI); Golgi body (G); lid (Ld). 1,300×.

the cytological level, the organisms differ in spore size. *M. nelsoni* spores are 6.8-11.4 μm long ($\bar{X}$ = 8.1) and 4.6-6.8 μm wide ($\bar{X}$ = 5.5) (Couch, 1967), whereas *Minchinia* sp. spores are 9.0-10.0 μm long ($\bar{X}$ = 9.6; N = 30) and 7.5-8.5 μm wide ($\bar{X}$ = 8.1; N = 30).

As opposed to the differences there were numerous similarities between the two species in nuclear, mitochondrial, spore Golgi body, and wall structure as well as in mitosis. Species distinctions are admittedly subjective, and it could be argued that the above differences are only apparent due to growth in different hosts. As with most species distinctions, subjectivity of necessity plays a dominant role, as in the present consideration, until adequate information is available. I have used spores from *Minchinia* sp. in attempts to infect oysters, but no infections occurred. However, since neither *Minchinia* spp. nor *Haplosporidium* spp. infections have been experimentally transmitted, this failure can not be considered as significant.

Ormières et al. (1973) proposed an interesting scheme to describe sporoplasm delimitation and spore wall formation in *Urosporidium jiroveci*. They suggested that there are possibly pairs of nuclei in the syncytium of cleaving sporonts in which one nucleus degenerates and the other nucleus becomes the spore nucleus. Upon subdivision of the syncytium into sporoblasts, they suggested that partial division of the sporoblast cytoplasm occurs in which the cytoplasm, associated with the degenerate nucleus, grows around the portion associated with the future spore nucleus. Continuity is believed to be maintained between the two portions of cytoplasm until later in spore maturation when complete cleavage occurs. They further suggested that the basic mechanism of sporoplasm delimitation may also occur in species of *Minchinia*. Such a mechanism could occur in *Minchinia* sp. following syncytium formation, but the evidence is inconclusive. It is true that developing spores are observed in which cup-shaped units of anucleate cytoplasm partially surround an uninucleate sporoplasm primordium; however, no cytoplasmic bridge was observed between the two and no degenerate nucleus was observed. Possibly cleavage of uninucleate sporoblasts occurs to yield the "cup" of cytoplasm and the sporoplasm as two distinct units followed by growth of the "cup" rim around the sporoplasm. Discontinuity between sporoplasm and extrasporoplasm cytoplasm is not difficult to accept early in spore morphogenesis, because it is well established that anucleate cells can carry on biosynthesis of macromolecules (Keck, 1969), thus synthesis of spore wall ornaments could possibly occur in anucleated cytoplasm of *Minchinia* sp. In addition, the sporoplasms of ascospores are delimited from the surrounding cytoplasm prior to wall formation, although wall formation does occur on the plasmalemma not in the cytoplasm surrounding the sporoplasm (Beckett et al., 1968; Carroll, 1969).

My observations of *M. nelsoni*, *M. costalis*, *M.* sp., and *U. crescens* further indicate that complete cleavage of the sporont frequently occurs to yield approximately spherical, uninucleate sporoblasts with no connection to a unit of cytoplasm which could be differentiated as an "envelope primordium" (see for example Fig. 12, Perkins, 1969). These spherical sporoblasts could cleave to form an anucleate "envelope primordium" as hypothesized by Ormières et al. (1973), but I have not seen evidence of it. Obviously the question as to how spores are formed will not be settled by observations of sectioned material from non-synchronously dividing cells. Living material or sections of synchronous cells fixed at known time intervals must be examined. Unfortunately it appears that the techniques and media required for culture of these organisms are unknown.

As I suggested earlier (Perkins, 1971), haplosporosomes appear to be characteristic of species of *Minchinia* and *Urosporidium*. Ormières et al. (1973) found them in *U. jiroveci* and now they have been found in *Minchinia* sp. Their function is unknown. Possibly isolation and subsequent biochemical characterization can be accomplished, thereby yielding an indication of their function. The ornaments or strands around the mature spore of *Minchinia* sp. are also consistent with the observations of the other species in that the spores of each species have one or two types of wrappings. These structures differ enough so that they could be used to distinguish the spores of the various species studied thus far except possibly *U. crescens* and *U. jiroveci* which seem to have the same structures.

Other workers such as Granata (1914), Pixell-Goodrich (1915), and Farley (1967) have either suggested or stated that karyogamy and subsequent meiosis are involved in sporulation in species of *Minchinia* and *Haplosporidium*, their chief evidence being the observations of paired nuclei and large variations in nuclear size. This study adds to those findings the observation of synaptonemal-like complexes and polycomplex-like structures as possible indicators of meiosis in sporonts. In addition, nuclear size changes during spore formation have been more thoroughly quantitated.

The morphogenetic sequence which appears to occur during sporulation is summarized in Figure 32. Plasmodia increase in mass and numbers of nuclei and form a delimiting wall at which time they are termed sporonts. The possibility that these increases result from plasmogamy of several plasmodia was not eliminated. Nuclear pairing follows, then karyogamy to form a brief diplophase in the life cycle, at which point nuclear sizes increase to about 4-6 μm diameter from about 2-4 μm. Meiosis results in reduction in nuclear size and a return to haplophase. The sporont then undergoes cytokinesis by one of two mechanisms. In one, the cytoplasm passes through a syncytial stage before grouping around nuclei to form uninucleate sporoblasts from which the sporoplasm is cleaved. In the other, both sporoplasms and sporoblasts are cleaved from the sporont forming free sporoblasts within the sporont wall and sporoplasms bound in the remaining cytoplasm of the sporont until subsequent cleavage frees them as sporoblasts with precleaved sporoplasm (Fig. 17). The latter mechanism is considered to be aberrant, because a large number of anomalously-formed spores are found in such sporonts; however, since spores, normal in appearance, are also observed and since the "aberrant" sporonts are frequently observed, they are believed to be a source of normal spores.

Since the proposed developmental sequence in Figure 32 is based on observations of non-living material, there are obviously many uncertainties concerning its accuracy. The evidence that karyogamy occurs during sporont de-

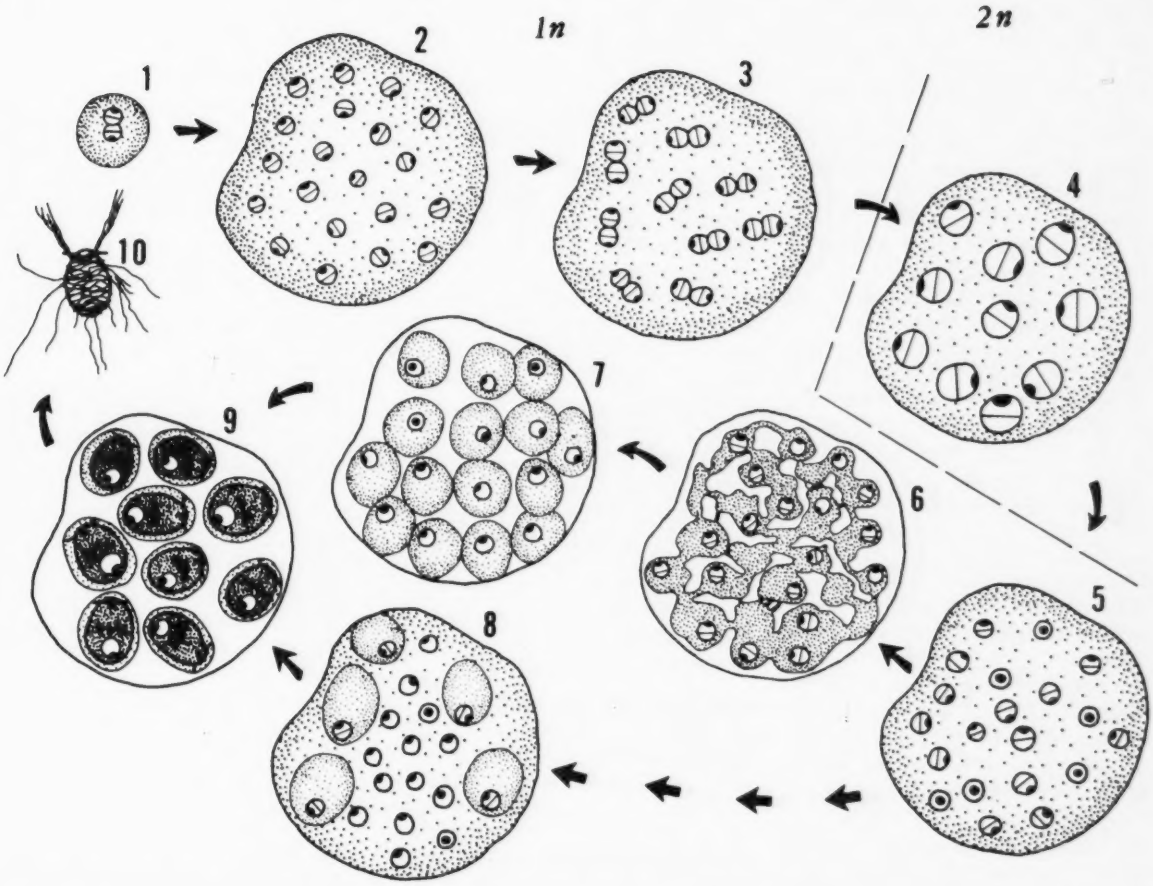


Figure 32.—Diagram of postulated sporulation sequence. Plasmodia (1) enlarge to form sporonts (2) (whether plasmogamy occurs is unknown) in which nuclei pair (3) then karyogamy results (4). The cells, therefore, pass from haplophase (1n) to diplophase (2n). Meiosis results in the return to haplophase and decrease in nuclear size (5). Cytokinesis occurs by one of two routes. In one a syncytium (6) is formed followed by condensation of cytoplasm around each nucleus to form uninucleate sporoblasts (7). In the other less significant pathway, sporoblasts or sporoplasms are cleaved from the sporont protoplast (8). Within sporoblasts cleavage occurs to yield sporoplasm surrounded by anucleate cytoplasm then a spore wall is formed at the interface of the two units (9) followed by spore maturation (10). Sporoplasms cleaved from the sporont protoplasm (8) are further delimited by cleavage of a unit of cytoplasm which surrounds them resulting in cells as seen in (9). The bars in nuclei represent the persistent mitotic apparatus.

velopment lies in the following observations: 1) a wide range of nuclear sizes (2.3-5.9 μm diameter) were observed in sporonts; 2) the larger nuclei were not the vesicular type thus are probably not the result of poor fixation as may be the case in plasmodia; 3) a pair of small nuclei (2.5 and 2.9 μm) were observed in a sporont with large nuclei (4.1-4.6 μm) (Fig. 9); 4) large (4-5.9 μm) nuclei were not paired.

Since large nuclei up to 8.0 μm were observed in plasmodia, there is a possibility that: 1) karyogamy occurs in plasmodia; 2) enlargement occurs before nuclear division; 3) there are two sexual strains with different size nuclei; or 4) enlargement is a fixation artifact. Either suggestions 1) or 3) could be correct, but there is not enough information to evaluate them at present. It is unlikely that marked nuclear enlargement is required before nuclear division in plasmodia, because anaphase and telophase division figures of different sizes were observed which correspond to the different sizes of interphase nuclei. The largest anaphase figure observed was 9.9 μm long × 5.1 μm wide and the smallest was 6.5 × 3.0 μm (N = 9). It is possible that enlargement could be the result of inadequate fixation, but this suggestion was not tested. Enlarged, vesicular nuclei have been noted by Haskin et al. (1966), Myhre (1969), and myself[3] in *M. nelsoni* plasmodia. As Myhre (1969) noted the large nuclei are "vesicular" with large unstained areas or spaces in the nucleoplasm. He noted also that nearly a full range of nuclei (2.0-7.5 μm) could be "vesicular," observations that I have verified for plasmodia of both *M. nelsoni* and *Minchinia* sp. After observations of thousands of *M. nelsoni* and hundreds of *Minchinia* sp. plasmodial nuclei fixed in glutaraldehyde and osmium tetroxide I have not seen the enlarged "vesicu-

[3]Perkins, Frank O. Fine structure of the haplosporidan *Kernstab*, a persistent, intranuclear mitotic apparatus. In preparation.

lar" nuclei. Only in material fixed in Davidson's, formol-alcohol, or Zenker's fixative were these nuclei seen. Small (<3.0 μm) vesicular nuclei were seen in the preparations for electron microscopy, but they were in moribund cells or associated with lysing oyster cells. Obviously, the matter of enlarged nuclei requires further examination.

As seen in Figure 10, attempts to clearly demonstrate two or three nuclear size classes were unsuccessful, possibly because of the inability to distinguish cell stages 2-5 (Fig. 32) (assuming that they exist). One would expect to see at least two modes if the small prekaryogamy (cell 2; Fig. 32) and post-meiotic (cell 5) nuclei were the same size and the post-karyogamy, large nuclei (cell 4) were in a different size range. A trimodal distribution would be expected if cells 2, 4, and 5 contained nuclei of different sizes. However, only a skewed distribution with one obvious mode was determined.

The evidence for meiosis lies in the observation of structures presumed to be synaptonemal complexes (SC's) and polycomplexes (PC's) (Figs. 11-13). Structures like those in Figures 11 and 12 are believed to be SC's because: 1) the structure and sizes are similar to SC's as previously described in other organisms (Perkins and Amon, 1969; Moens and Perkins, 1969; Wettstein and Sotelo, 1971); 2) they were found only in sporonts and were infrequently observed; 3) they were not present in paired nuclei; and 4) they were absent from nuclei, obviously in anaphase or telophase, as evidenced by nuclear membrane profiles. The chief difference between the present complexes and PC's reported from other organisms, such as the water mold *Lagenidium callinectes* (Amerson and Bland, 1973) or the mosquito *Aedes aegypti* (Roth, 1966), is the absence of medial elements in *Minchinia* sp. complexes. If the structures are PC's, possibly they represent degenerate forms in which the medial elements were lost or not preserved during fixation. Some PC-like structures in *Minichinia* sp. more closely resembled those of *L. callinectes* in that the dense bands were slightly bent in a shallow V-shaped or curved configuration.

I suggest that meiosis occurs in a nearly synchronous manner within each sporont (Fig. 14) to yield sporonts with nuclei of a size approximating those of prekaryogamy sporonts and those just before meiosis (Fig. 15). It was possible to identify late sporonts by the presence of incomplete cytoplasmic cleavage furrows. It is unlikely that cleaving plasmodia were misidentified as sporonts, because 1) plasmodia are much smaller and have fewer nuclei, 2) the sporont wall was visible on the cleaving cells, and 3) plasmodia were not observed to subdivide into uninucleate cells.

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MFR PAPER 1148

A Brief Review of the Involvements of *Lagenidium*, an Aquatic Fungus Parasite, with Arthropods

CLYDE J. UMPHLETT and E.M. McCRAY, JR.

ABSTRACT—Several species of the genus Lagenidium, *an aquatic planomycetous fungus, have been reported as parasites of arthropods including crabs, barnacles, and mosquito larvae.* Lagenidium callinectes, *a superficial parasite of egg masses of the blue crab, has on some occasions been found on as many as 40 percent of the egg masses collected from Chesapeake Bay waters. The same fungus species has been found as a parasite in the ova of the barnacle* Chelonibia patula *in waters off the North Carolina coast. A second species,* Lagenidium chthamalophilum, *has been observed in 34 percent of the gill lamellae of the barnacle* Chthamalus fragilis. *A* Lagenidium *sp. has been observed in laboratory-reared brown shrimp,* Penaeus aztecus, *and white shrimp,* Penaeus setiferus. Lagenidium giganteum *has been shown to be a virulent pathogen of larvae of several species of culicine mosquitoes including* Aedes aegypti, *with over 90 percent of test larvae in laboratory experiments killed consistently.* Lagenidium giganteum *has been shown to be an effective larval pathogen under field conditions also, but does not appear to be as effective against anophelines as against culicines.*

Interest in environmental conservation in recent years has prompted a search for means of controlling populations of noxious organisms with other than chemical pesticides, and there is interest of long standing regarding diseases occurring in populations of desirable and profitable organisms such as certain crustaceans. Out of these two interest areas has arisen a small literature dealing with certain aquatic fungi among which is the genus *Lagenidium*. This aquatic fungus has a long history of parasitism, and several species have been recorded from a variety of hosts including algae, other fungi, certain insects, and some of the lower and higher crustaceans (Sparrow, 1960). Species of *Lagenidium* have been found in hosts from both freshwater and marine habitats.

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CRUSTACEAN INFECTIONS

Lagenidium callinectes was described by Couch (1942) as being parasitic in ova of the blue crab, *Callinectes sapidus*. Johnson and Bonner (1960) reported the occurrence of the same fungus on lamellae of the barnacle, *Chelonibia patula*. Couch described the fungus mycelium as being intracellular in the crab ova, whereas Johnson and Bonner found that the preponderance of fungal hyphae in the barnacle was extramatrical. They concluded, however, since the fungus transferred readily from barnacle to blue crab eggs in cross inoculation experiments that the fungus on the barnacle was indeed *Lagenidium callinectes*. In a comprehensive study of the disease caused in blue crabs by *L. callinectes*, Rogers-Talbert (1948) described the fungus as a peripheral parasite of the egg masses. She noted that the eggs were susceptible to infection in all stages of development. The spread of the fungus over the sponge was rapid, but it usually appeared to penetrate no deeper than three millimeters. Development of the eggs at the interior of the sponge was not retarded by the infection. Heavily diseased sponges were infected to the extent that about 25 percent of the eggs in the mass contained the fungus, and in a given sample of experimental crabs some 80 to 90 percent exhibited some degree of infection. Development of the fungus was rapid at salinities between 5 and 30 ppt, but abnormal development was noted in fresh pond water. Rogers-Talbert observed also that eggs of the oyster and mud crab were attacked in the laboratory under conditions favoring very rapid transmission of the infection. Scott (1962), in a survey of the phycomycetous fungi of marine and brackish waters in the vicinity of Gloucester Point, Va., reported that 40 percent of the blue crab egg masses collected were infected with *Lagenidium callinectes*. Bland and Amerson (1973) surveyed over 2,000 ovigerous crabs during the summer of 1971 and obtained isolates of *L. callinectes* with which they performed a detailed morphological study, but did not report the extent of the fungus in the crab population.

Another marine species has been described by Johnson (1958). *L. chthamalophilum* in the barnacle *Chthamalus fragilis* was reported in 34 percent of all host lamellae inspected. This percentage of infection was based on hosts collected from piling and mooring stakes, since 86 barnacles of the same species collected from salt marsh cord grass exhibited only three infections with *L. chthamalophilum*. Attempts to infect the barnacle, *Balanus amphitrite*, with fungus material from *C. fragilis* were unsuccessful.

Lightner and Fontaine (1973) recently observed that a *Lagenidium* sp. was infective to larval white shrimp, *Penaeus setiferus*, and a brown shrimp, *Penaeus aztecus*, reared under laboratory conditions. Natural mortality occurred in 12.4 percent of the shrimp after the fungal mycelium had invaded and replaced nearly all the internal tissues, while 20.0 percent of the larval shrimp died after experimental exposure to the fungus.

INFECTIONS IN OTHER ARTHROPODS

Couch (1935) described in North Carolina the only species of

Figure 1.—(*Lagenidium giganteum* parasitizing *Culex restuans*): Non-septate hyphae growing in abdominal hemicoele. 125×.

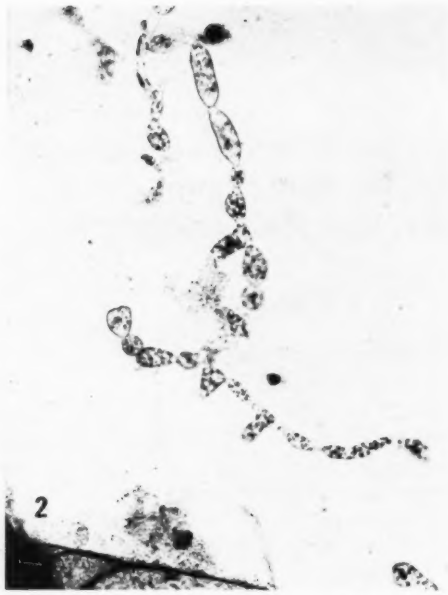

Figure 2.—(*Lagenidium giganteum* parasitizing *Culex restuans*): Septate hyphae dissected from host; each cell is potentially a sporangium, 125×.

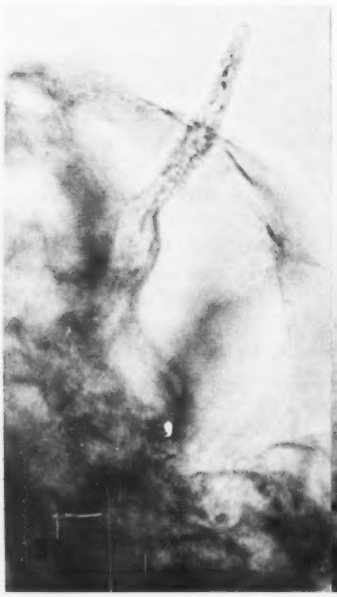

Figure 3.—(*Lagenidium giganteum* parasitizing *Culex restuans*): Discharge tube forming from sporangium and penetrating exoskeleton of host cadaver. 600×.

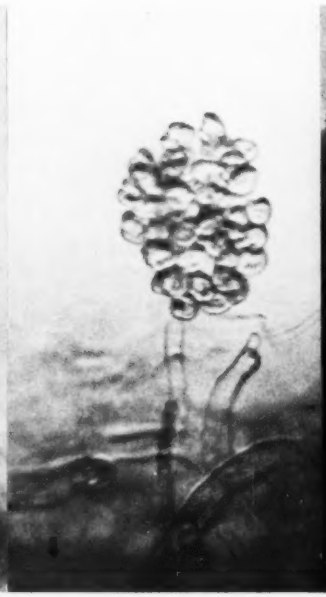

Figure 4.—(*Lagenidium giganteum* parasitizing *Culex restuans*): Biflagellate zoospores swarming in vesicle just prior to release. 600×.

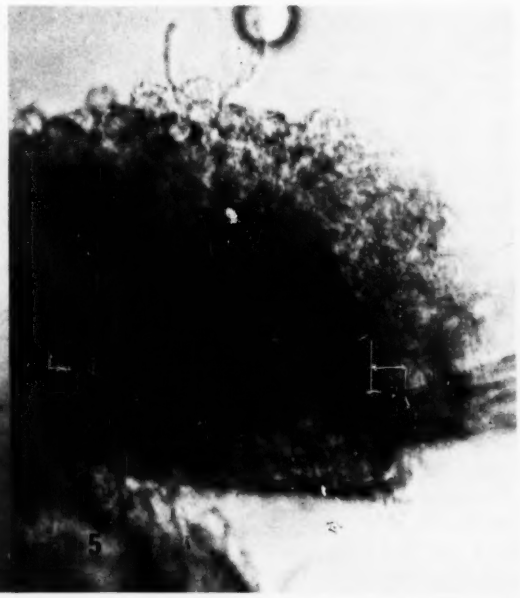

Figure 5.—(*Lagenidium giganteum* parasitizing *Culex restuans*): Cluster of encysted zoospores on anal segment; infection initiated here. 600×.

Lagenidium reported thus far to occur as a parasite in mosquito larvae. *L. giganteum* was described as a saprophyte which could function as a weak, facultative parasite of culicine mosquitoes. Willoughby (1969) reported on the nutrition of a saprophytic strain of this species which he had isolated on termite wings from a stream in England, but he did not cite any parasitic relationship of his isolate with mosquito larvae. One of the authors (CJU) isolated into pure culture two strains of what was apparently *L. giganteum* in 1963. One of the strains originated from a parasitized culicine larva, the other from a parasitized anopheline larva. Both were from Orange County, N.C. A very brief and unreported test of the fungus strain from the culicine larva at that time indicated that the fungus could

infect larvae of *Aedes aegypti*, but this line of work was not pursued until 1969 when an isolate of *L. giganteum* was obtained from a culicine larva from one of the original habitats.

An infection of a mosquito by *L. giganteum* results in the development of mycelium consisting of narrow, branching hyphae (Fig. 1) which soon increase in diameter and become septate. The hyphal segments resulting from the septations swell, thereby producing hyphae that are constricted at the septa (Fig. 2). Within 72 h after infection has occurred, the coelomic cavity of the larva is about filled with mycelial growth, and in many instances hyphae can be seen growing in the aorta of the insect. Death of the larva occurs at this time. About 24 h after an infected larva is dead, zoospore production is initiated by the fungus. The hyphal segments produce thin discharge tubes that penetrate the exoskeleton of the dead insect (Fig. 3). Through these tubes the cytoplasm contained in the segments is discharged to the outside where it is retained for a few minutes in a membranous vesicle. Cleavage of the cytoplasm occurs in the vesicle, and the biflagellate zoospores formed there escape when the vesicle breaks down (Fig. 4). The zoospore is the infectious agent (Fig. 5).

In the first report of experimentation with *L. giganteum* against mosquito larvae, Umphlett and Huang (1972) noted that this isolate behaved as a virulent parasite of *C. restuans* in laboratory tests. They found that the level of infection in larval populations varied with the amount of inoculum which was supplied as zoospores. Over 90 percent of 4-day-old larvae subjected to ca. 0.5 million zoospores (3 units) per larval culture were killed within 72 h after inoculation, whereas 10-day-old larvae with the same quantity of inoculum were stricken only at a 5 percent level (Fig. 6). However, in tests using ca. 1.5 million zoospores (9 units) per larval culture over 90 percent of larvae at all ages tested up to 10 days were killed (Fig. 6). It was noted also that when the host population was doubled and held in the same size container, larval mortality was three times that of the control when 0.5 (3 units) or 1.0 million zoospores (6 units) were utilized. When 1.5 million zoospores (9 units) were applied, mortality above 90 percent prevailed in all tests regardless of host density or larval

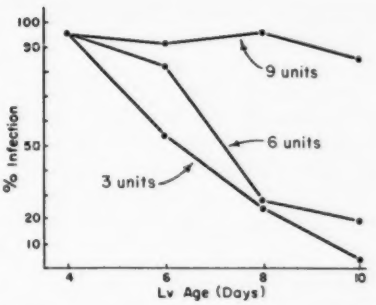

Figure 6.—Effect of larval (Lv) age in *Culex restuans* on the quantity of *Lagenidium giganteum* inoculum required to kill larvae. Note that 1.0 million zoospores equals 6 units. From Umphlett and Huang, 1972.

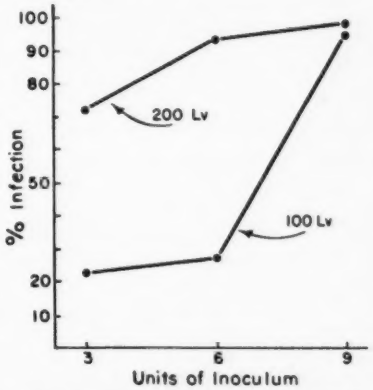

Figure 7.—Effect of larval (Lv) population density (*Culex restuans*) on the level of infection by *Lagenidium giganteum* at various concentrations of inoculum. Note that 1.0 million zoospores equals 6 units. From Umphlett and Huang, 1972.

age (Fig. 7). In a short preliminary field test, Umphlett and Huang (1972) reported that 43 percent of the larvae of *C. restuans* collected from the experimental pool three days after the introduction of inoculum were infected, 8 percent of the *Anopheles* sp. larvae in the same collection were infected, and 4 percent of the larvae of *Psorophora* sp. were infected with the test fungus. Larvae of *Anopheles* sp. occurred in three subsequent collections, but *C. restuans* larvae did not appear in any sample after the first. Umphlett and Huang (1972) suggested that *L. giganteum* was not strikingly effective against *Anopheles* spp. McCray, Umphlett, and Fay, (1973) subsequently corroborated this by reporting no mortality in *Anopheles* spp. tested. However, Giebel and Domnas (In press) reported that they were able to obtain up to 85 percent mortality of *Anopheles quadrimaculatus* in some tests, but remarked that in some experimental series no infection or only a low rate, from 5 to 10 percent, was obtained. McCray, Umphlett, and Fay, (1973) extended the known host range of *L. giganteum* to include *Aedes aegypti*, *Ae. mediovittatus*, *Ae. taeniorhynchus*, *Ae. triseriatus*, *Ae. sollicitans*, *Culex quinquefasciatus*, *Cu. tarsalis*, *Cu. fatigans*, and *Cu. nigripalpus*.

In a recent small field test with *L. giganteum* (McCray, Womeldorf, et al., 1973), two distinctly different habitats in California were utilized. The site near Hanford was intermittently dry and flooded irrigated pasture land in which *Aedes nigromaculis* was the principal mosquito species present. The fungus was applied in the test area by spraying sporangia into the water from a back-pack sprayer. To every square foot of water surface a number of sporangia approximating the number produced by the fungus in one infected fourth instar larva was applied, a potential of about 250,000 zoospores per square foot. Infection of the natural populations of *Ae. nigromaculis* in the test areas did occur, and all infected specimens died. Field populations were dramatically reduced within three days after treatment (Table 1). At this same test site, larvae of *C. tarsalis* appeared in the treated areas subsequent to the test. These larvae became infected by the fungus, and all animals collected were found to be infected and subsequently died.

The second study site, near Colusa, Calif., was in the vicinity of rice fields and associated drainage ditches. The test sites were not in the rice fields proper, but rather were isolated ditches nearby. *C. tarsalis* was the target organism in this area and three experimental sites were chosen. The water in Site No. 1 contained a high level of dissolved solids and had a pH of 10.0, while Site No. 2 had a pH of approximately 8.0, and water qualities here resembled those of the rice fields and drainage

Table 1.—The number of living *Aedes nigromaculis* larvae collected and found infected following introduction of *Lagenidium giganteum* at the site near Hanford, Calif., 1972. From McCray, Womeldorf, et al., 1973.

Sampling plot	Day of treatment	Post-treatment days 3	4
Test 1	411	0	0
Test 2	321	3[1]	0
Test 3	309	0	0
Control 1	367	75	24

[1]All three larvae died and were infected with *Lagenidium*.

ditches. At Site No. 3 the chloride ion concentration was about 25 times that of the normal habitat in which *C. tarsalis* breeds. Table 2 shows the number of living larvae of *C. tarsalis* collected and found infected following the introduction of *L. giganteum* in Sites 1, 2, and 3. It can be seen that in Site 2, which most nearly resembled the normal breeding habitat of the mosquito, a single introduction of the fungus infected and eliminated the natural population of *C. tarsalis*. The effect of the fungus on mosquito larvae was reduced, though, in Sites 1 and 3 in which water analyses had revealed conditions known to be detrimental to the fungus. Table 3 shows the mean daily pre- and post-treatment collections of living *C. tarsalis* larvae and pupae from Site 2 inoculated with *L. giganteum*. It should be noted that on the fifth post-treatment day no living larvae or pupae were collected, and none appeared as late as the seventeenth post-treatment day when the test was terminated.

During these studies more than 1,400 aquatic non-target organisms (small crustaceans and insects) from the treated sites were examined. No infection was observed in any of these specimens. Results of recent pathogenicity tests using *L. giganteum* at the Center for Disease Control, Atlanta, Ga.,[1] indicate that the fungus is not pathogenic to small mammals.

Umphlett and Huang (1972) offered the opinion that there is sufficient promise to dictate that further studies aimed at realization of the full potential of *L. giganteum* as an agent for the biological control of mosquitoes are feasible and desirable. McCray, Womeldorf, et al. (1973) stated that their studies revealed that the Umphlett strain of *L. giganteum* is an excellent candidate for further evaluation as a biological control agent, and that more definitive tests are in order.

[1]Ajello, L. Chief, Medical Mycology Section, Center for Disease Control, Atlanta, Ga. Pers. commun.

Table 2.—The number of living *Culex tarsalis* larvae collected and found infected following introduction of *Lagenidium giganteum* at sites near Colusa, Calif., 1972. From McCray, Womeldorf, et al., 1973.

Day after treatment	2	3	4	5	Total
Site #1					
Larvae collected	388	399	206	198	1,191
Larvae infected	0	100	3	2	105
Percent infected	0	25.5	1.4	1.0	8.8
Site #2					
Larvae collected	146	101	8	0	255
Larvae infected	146	101	8	-	255
Percent infected	100	100	100	-	100
Site #3					
Larvae collected	114	81	45	46	286
Larvae infected	0	15	3	4	22
Percent infected	0	18.5	6.7	8.7	7.6

Table 3.—Mean daily pre-and post-treatment collections of living *Culex tarsalis* larvae and pupae from Colusa site #2 inoculated with *Lagenidium giganteum*. From McCray, Womeldorf, et al., 1973.

Day	−4	−3	−2	−1	0[1]	+1	+2	+3	+4	+5	+17
Control[2]	110	122	102	125	88	72	93	80	123	112	111
Test[3]	96	96	78	93	89	88	51	36	5	0	0

[1]Day of inoculation.
[2]All instars from two plots combined.
[3]All instars from three plots combined.

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